Regulation of Hepatic Transport in Experimental Cholestasis

Nynke R. Koopen

Regulation of Hepatic Transport in Experimental Cholestasis

Stellingen behorend bij het proefschrift

Mechanisms involved in malabsorption of dietary lipids

Mini Kalivianakis
Groningen, 23 september 1998

Severe fat malabsorption due to impaired lipolysis can be identified by the ^{13}C-MTG breath test. However, in situations of mild fat malabsorption, considerable interindividual variation in the results of the ^{13}C-MTG breath test occurs, which may be explained by a shift in the rate-limiting step in the overall process of $^{13}CO_2$ production.
(DIT PROEFSCHRIFT)

The continuing fat malabsorption in cystic fibrosis patients on enzyme replacement therapy is not likely due to insufficient lipolytic enzyme activity, but rather due to either incomplete intraluminal solubilization and/or reduced mucosal uptake of long-chain fatty acids.
(DIT PROEFSCHRIFT)

Vrouwelijke wetenschappers moeten ongeveer 2½ maal beter presteren dan hun mannelijke collega's voor een vergelijkbaar resultaat.
(NATURE 1997;387:341)

Een promotieonderzoek is niet alleen een investering is in jezelf maar ook in de universiteit. Dat dit besef ook begint door te dringen tot de universitaire wereld blijkt uit het feit dat een aantal universiteiten bereid is de promovendi meer te betalen.

Mini staat voor meer dan klein.

Ook voor stellingen geldt dat een hogere kwantiteit vaak niet ten goede komt aan de kwaliteit.

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RIJKSUNIVERSITEIT GRONINGEN

REGULATION OF HEPATIC TRANSPORT IN EXPERIMENTAL CHOLESTASIS

Proefschrift

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Yn in om en omsjoch

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Contents

Chapter 1

General Introduction

GENERAL INTRODUCTION

Cholestasis a clinical condition in which the formation of bile is impaired. The causes underlying the development of cholestasis in patients can be extremely varied. This thesis describes the relationship between bile production and composition, levels and activity of specific hepatic transport proteins involved in bile formation and the diagnostic value of cholestatic serum markers like bile salts and bilirubin in different experimental models.

1. Bile formation

The formation of bile is an important function of the liver. Bile contains bile salts, phospholipids cholesterol, a variety of proteins and bilirubin as its main organic constituents. Electrolytes and a number of trace elements are also present in bile. Bile serves many functions in the body. In the first place, the biliary pathway represents the major route for elimination of endogenous and exogenous waste products. Bilirubin, which gives bile its bright yellow colour is produced during the breakdown of hemoglobin from senescent red blood cells and of other heme-containing proteins and is secreted into bile after the coupling of sugar groups, which renders this hydrophobic compound more water-soluble. Bile also plays a role in disposition from the body of a wide variety of xenobiotics, including drugs and heavy metals. Secondly, bile formation is important in the maintenance of cholesterol homeostasis. Cholesterol leaves the body almost exclusively after its excretion into bile, either as the free compound or after its conversion to bile salts. Thirdly, bile is essential for the solubilization by bile salts of dietary lipid and lipid-soluble vitamins in the small intestine and for the assembly of chylomicrons by the enterocytes: both processes are required for efficient intestinal absorption of dietary fats and fat-soluble vitamins. Therefore, maintenance of bile formation is not only essential for functioning of the liver but also of the organism as a whole.

Bile is formed by the liver parenchymal cells, or hepatocytes, which represent the major cell type in the liver in quantitative terms (1). Hepatocytes are polarized cells with their basolateral or sinusoidal membrane facing the blood and their apical or canalicular membrane facing the bile (figure 1). To maintain this polarization, the canalicular domain of adjacent cells is sealed from the blood compartment by means of tight junctions, through which large molecules can not pass, but which are permeable for electrolytes and water. In order to be secreted into bile, most compounds therefore have to pass through the hepatocytes. The hepatocytes are arranged in one cell thick layers that line the sinusoids in the liver-lobules, the smallest structural units of the liver according to the model of Rappaport (figure 2). This lobule receives blood via branches of the portal vein and the hepatic artery. Via the sinusoids the blood then flows to the central vein. Along the sinusoids there is a functional division of the hepatocytes into three zones, i.e. the periportal, the central or intermediate and the perivenous zone. A morphological and functional heterogeneity of hepatocytes exists along these different zones with respect to metabolic- and transport functions (2,3).

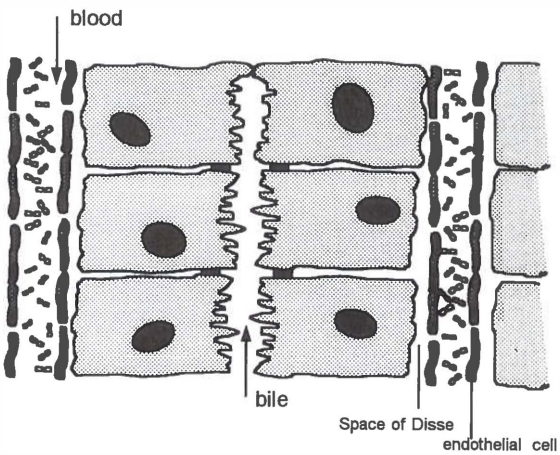

Figure 1. Schematic representation of liver cell plate. *Hepatocytes are polarized cells. Proteins and solutes from the blood pass the endothelial barrier, whereas erythrocytes and other blood derived cells cannot pass. Hepatocytes absorb compounds from the space of Disse. These compounds and waste products generated inside the liver are secreted in the canaliculus. Bile leaves the lobule in the direction opposite to blood flow. The sinusoidal and canalicular compartment are separated by tight junctions.*

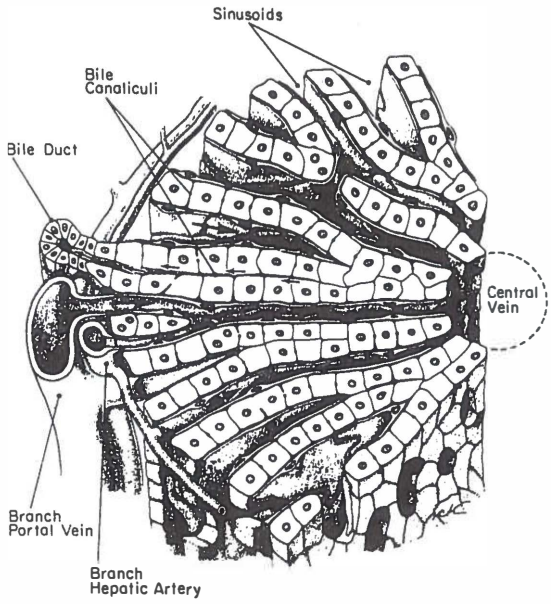

Figure 2. Schematic representation of the liver lobule. *Hepatocyte cell plates are radially arranged around the central vein to form a hexagonal shaped liver lobule. Blood enters the lobule via branches of the portal vein and branches from the hepatic artery that combine to form the sinusoidal blood flow (large arrow). Bile leaves via the bile ducts. (small arrow). Adapted from Bloom et al. (1975) A textbook of histology.*

The canalicular membrane surrounds the bile canaliculus, a small channel formed between adjacent hepatocytes. These canaliculi end up in the small bile ductules that, in turn, drain the larger bile ducts. These bile ducts join to form the common bile duct which ends up in the gallbladder in humans and mice. Other species, like the rat, do not have a gallbladder. Via the common bile duct or from the gallbladder, bile is expelled into the intestine after ingestion of a (fat-containing) meal.

The canalicular membrane is the site were bile components are actively transported into the canaliculus by specific transporters. These transport processes create an osmotic force that drives water and some ions from the blood via the tight junctions into the canaliculus. This osmotic force comprises two major components. The first, the bile salt-dependent fraction of bile flow (BSDF), is generated by the secretion of bile salts into the canaliculus. The relationship between bile salt secretion and bile flow is a linear one (Figure 3).

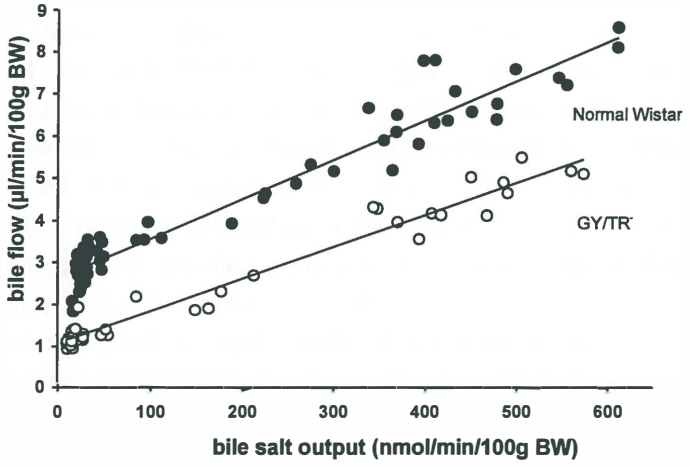

Figure 3. Relationship between bile flow and biliary bile salt secretion. *Rats were equipped with permanent catheters in bile duct and duodenum and both catheters were immediately connected to each other to maintain the enterohepatic circulation. Eight days after surgery the enterohepatic circulation was interrupted and bile was collected in 30 min. fractions, in both normal Wistar and mutant GY/TR⁻ Wistar rats. The bile flow at the hypothetical zero value of bile salt secretion represents the bile salt independent fraction of bile flow. This BSIDF is decreased in GY/TR⁻ rats, in a part caused by reduced biliary GSH secretion in these mutant rats.*

A second component of bile flow becomes apparent when this linear relationship is extrapolated to a hypothetical zero bile salt secretion. This fraction represents the so-called bile salt- independent fraction of bile flow (BSIDF) of which, until now, the nature is not completely defined. However, a major part of this fraction can be explained by secretion of glutathione (4,5). Mutant rats, that lack the

ability of glutathione secretion into bile, show a decreased BSIDF (6,7) (figure 3). Another determinant of this BSIDF is the secretion of HCO_3^- into the canaliculus (8). A more detailed description of bile formation process, including the recently proposed role of bile duct epithelial cells or cholangiocytes, can be found in chapter 10. In order to maintain BSDF, which represents the major fraction of bile in most animal species, the bile salts are efficiently kept within an enterohepatic circulation. After their secretion into the intestinal lumen, bile salts are taken up by the enterocytes and transported back to the liver via the portal vein in order to be secreted again.

2. Bile salt synthesis

Although bile salts are efficiently conserved in the enterohepatic circulation, a small fraction (about 5%) escapes intestinal reabsorption and ends up in the feces. Because of the frequent cycling (about 8 times /day) of the pool this fecal loss amounts up to 1 gram per day in humans (9). In order to maintain the bile salt pool, this loss has to be compensated for by *de novo* synthesis, which takes place in the hepatocytes, primary in those localized in the pericentral areas (10).

The primary bile salts are synthesized from cholesterol via a sequence of enzymatic conversions (11-14) (Figure 4). In most mammals the two major primary bile salts are cholate and chenodeoxycholate (15). Among different species however, the biliary bile salt composition shows a marked variety of differently structured bile salts. For instance, β-muricholate is a major biliary bile salt in rats and mice that is formed from chenodeoxycholate in the liver (15), in contrast to humans. Bile salt structure can also be modified by interactions with intestinal bacteria leading to the formation of so-called secondary bile salts (9). In addition, bile salt structure can be modified again, after returning to the liver, leading to formation of tertiary bile salt species (9). The very recently identified Δ^{22} β-muricholate (16) present in rodent bile represents an example hereof (see chapter 5). In this way, a wide diversity in bile salt species can be formed. The composition of the bile salt pool varies widely among different animal species as well as in different pathological states (17). Prior to their secretion in bile, bile salts are conjugated with either glycine or taurine. This conjugation is important for efficient secretion of bile salts into bile and for their role in lipid digestion (18). Regulation of bile salt synthesis has been extensively reviewed in the past few years (12,13): a profound overview is beyond the scope of this introduction. It is known that bile salts present in the pool regulate key enzymes in their own biosynthetic pathways (12,13). Interruption of the enterohepatic circulation in rats leads to increased activity and mRNA levels of both the cholesterol 7α-hydroxylase and sterol 27-hydroxylase (see chapter 5), catalyzing the first steps of the pathways involved in bile salt synthesis, i.e. the neutral pathway and the acidic or alternative pathway (19). The regulation of sterol 27-hydroxylase is not yet clear as is also the case for the regulation of sterol 12α-hydroxylase, the enzyme linking the neutral and acidic pathways.

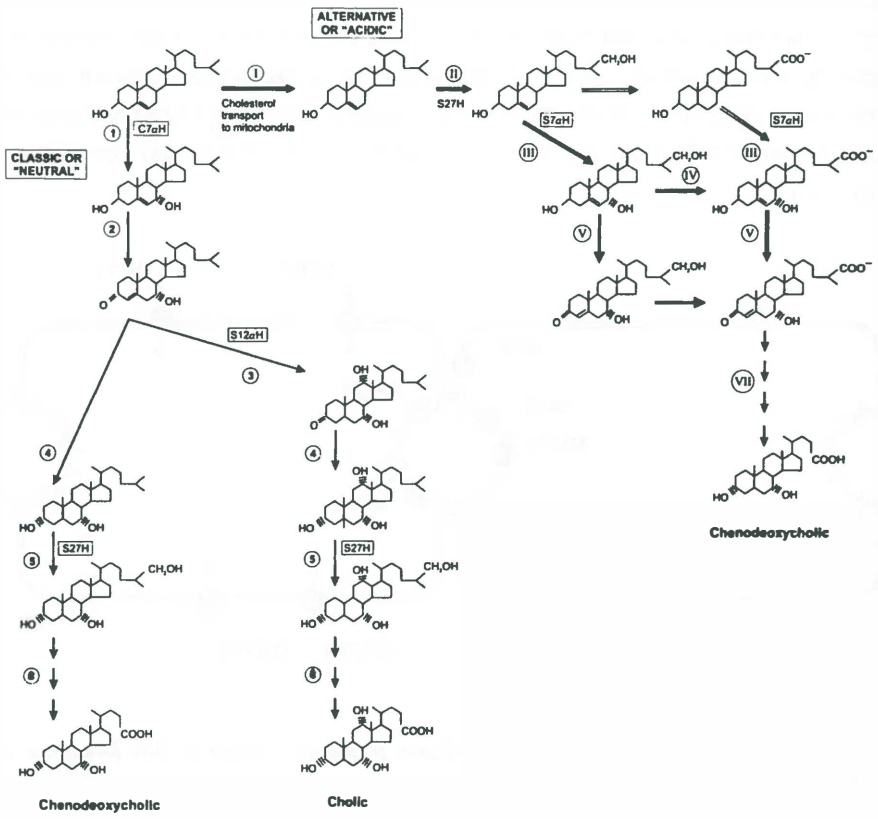

Figure 4. Bile salt biosynthetic pathways. *Microsomal cholesterol 7α-hydroxylase and mitochondrial sterol 27-hydroxylase catalyze the initial reactions in the neutral and acidic pathway, respectively. The endproducts of the neutral pathway are cholate and chenodeoxycholate and chenodeoxycholate is the main product of the acidic pathway.*

It has recently become clear that hepatic cholesterol synthesis mainly takes place in periportal cells (20,21). In contrast, bile salt synthetic enzymes are localized predominantly pericentrally (10), indicating that under normal circumstances these processes are functionally separated. The liver is, however, able to efficiently regulate the formation of bile salts in response to the demand by expanding the expression of key enzymes to a larger area within the liver acinus, thereby physically linking bile salt synthesis to cholesterol synthesis (12,22).

3. Hepatic membrane-bound transport systems

In order to maintain the enterohepatic circulation of bile salts and to allow for the transport of a wide variety of other endogenous and exogenous compounds, the liver contains a great number of solute-transporting proteins (23,24) (figure 5). The activity of uptake systems depends largely on a transmembrane ion-gradients whereas secretion is, in general, directly dependent on energy provided by the hydrolysis of ATP. In the past few years a number of these systems have been identified and characterized.

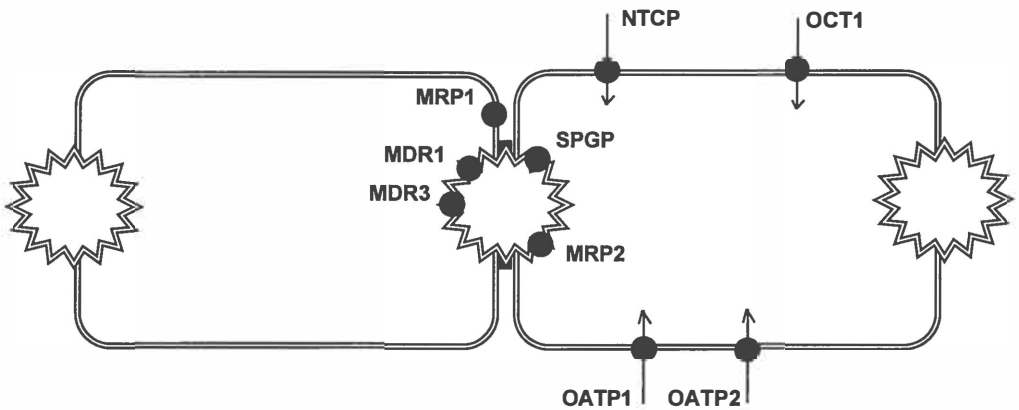

Figure 5. Current concept of carrier mediated transport involved in bile formation in humans.

3.1 Sinusoidal transport systems

Bile salts are actively taken up from the blood by the liver via a sodium-dependent transport system. This transport protein transports bile salts into the cell in co-transport with sodium (25,26). The Na^+-gradient required for this transport is generated by an ATP-dependent counter-transport with K^+, by the Na^+/K^+ ATPase abundantly present in the sinusoidal membrane (25). In this process, two sodium molecules are exchanged for 3 potassium molecules in order to create a sodium gradient (27). The so-called Na^+-dependent taurocholate transporter (ntcp) has been cloned from human, rat and mouse liver (28-30). In rats, this protein is a 362 amino acid glycoprotein with seven transmembrane domains with an apparent molecular mass of 51 kDa. Ntcp is localized exclusively in the sinusoidal membrane (31). There is no zonal heterogeneity in expression of ntcp (31). This protein mediates sodium-dependent transport of conjugated bile salts such as taurocholate and is thought to be responsible for the predominant fraction of hepatic bile salt uptake (25,26). The expression of ntcp is strongly dependent on the differentiation state of the hepatocytes. Ntcp disappears rather fast in primary cultured hepatocytes (32), is not expressed in hepatoma cell lines (32,33) and is down-regulated in regenerating livers (34,35). In cholestatic models, including bile duct ligation, ethinylestradiol-

treatment and sepsis, ntcp protein- and mRNA levels are decreased (36-40). During ontogeny, ntcp appears relatively late, i.e. at days 18-21 of gestation. After birth ntcp levels gradually increase, reaching adult levels within a few weeks (41). Recently, genomic cloning and analysis of the 5' upstream DNA sequence of the rat *ntcp* gene has been performed. The gene is localized on chromosome 6q24 and organized in five exons separated by four introns (42). Three elements within the promoter were shown to be related to the expression of ntcp: a TATA element, a hepatocyte nuclear factor (HNF1) binding site and a recently discovered palindromic recognition-site for an unknown liver-enriched factor (42). Furthermore, *ntcp* contains a possible bile salt responsive element, which is identical to the one found in the cholesterol 7α-hydroxylase gene, probably providing the key to regulation by its substrates, the bile salts. The exact mechanisms involved in regulation of ntcp remain to be determined.

A second organic anion transporter has been cloned from rat and human liver, called the organic anion transporting protein or oatp (43,44). Its function is not properly understood yet. The rat oatp 1 is a multispecific transporter which is able to accommodate a wide variety of structurally unrelated and differently charged amphiphatic molecules including bile salts (26,44) in a sodium-independent fashion. Oatp is able to transport bi-directionally, suggesting it to play a role as an overflow pump in pathological conditions associated with cholestasis (25). It is thought that different oatp isoforms exist in the liver with different substrate specificities (26). Recently, a second member of the rat oatp family has been cloned (oatp-2), with a high expression in liver, brain and kidney, and which has a similar substrate specificity as oatp-1, but in addition has a high affinity for digoxin (45).
Organic cations, for example drugs with tertiary or quartinairy amine groups, or organic cations like choline, thiamine and N-nicotinamide are likely to be transported by the organic cation transporters such as oct-1, a transporter cloned from a rat kidney cDNA library (46). This transporter shows no homology with the oatp's but also transports in a sodium-independent fashion (46).

3.2 Intracellular transport

Following sinusoidal uptake, biliary compounds have to pass trough the hepatocyte in order to be secreted at the canalicular membrane. The transcellular transport of organic anions including bile salts is thought to include both the binding to small cytosolic binding proteins and vesicle-mediated processes, the latter in particular during high substrate loads (47,48). Bile salts are known to bind to cytosolic proteins such as 3α-hydroxysteroid dehydrogenase and are transported to the canalicular membrane primarily by diffusion (49-51). The binding to cytosolic proteins retains bile salt in the cytoplasm in order to minimize reflux into plasma and redistribution to other cell compartments. Inhibition of this binding delays biliary bile salt secretion (52,53). The importance of vesicle mediated transport processes is not quite clear. It was postulated that about 10% of total bile flow under basal conditions is accounted for by a transcytotic pathway (54). A close relation between the trancytosis of protein markers like horseradish peroxidase (HRP) and bile salt transport has been observed (54-56).

This transport involves endocytosis at the sinusoidal membrane, microtuble-dependent intracellular vesicular transport and exocytosis at the canalicular membrane (54) Both bile salt transport and transport of some organic anions is inhibited by cholchicine, an inhibitor of microtuble-dependent transport (48,57). However, "packaging" of bile salts into vesicles has never been documented. (58,59). A sodium-independent transport system for bile salts has been identified in ER vesicles but its physiological role remains unclear (60).

An alternative explanation for the dependency of bile salt and organic anion transport on microtubule function can be derived from several studies. Hubbard and colleagues have provided evidence that after their synthesis in the Golgi compartment, canalicular proteins are first transported to the basolateral membrane and subsequently, via a transcytotic pathway, to the canalicular domain (61-63). Oude Elferink *et al.* provided evidence for recycling of the multispecific organic anion transporter to and from the canalicular membrane (64). In isolated hepatocyte couplets it was shown that secretion of an fluorescent bile salt into the canalicular space is stimulated by dibutyryl cAMP and that this effect could be abrogated by microtubule disruption (65). Furthermore, it was found that this stimulation was accompanied by increased canalicular membrane surface area (65,66). It was proposed that microtuble-dependent insertion into the canalicular membrane of pericanalicular vesicles containing transporters is responsible for this phenomenon, explaining the dependency of transport on microtuble action (48,59,65). Under cholestatic conditions, like bile duct ligation, this process is disturbed resulting in an increased number of these pericanalicular vesicles (67,68).

3.3 Canalicular transport systems

The canalicular membrane comprises only 10 % of the total hepatocyte membrane surface (69). The secretion across the canalicular membrane is thought to be the rate-limiting step in vectorial hepatobiliary transport. For most compounds secreted into bile, this step involves transport against a large concentration gradient between hepatocyte and bile. This concentration gradient is too large to be accounted for by membrane-potential driven transport only (69). It has become clear in the recent past that most of these transport systems are in fact ATP-driven and belong to the ATP Binding Cassette (ABC) transporter superfamily (70-72). This group of proteins comprises one of the largest superfamilies in both prokaryotes and eukaryotes and can be subdivided into a number of sub-families. Within the context of this thesis, the P-glycoprotein (Pgp) and the Multidrug Resistance Protein (MRP) subfamilies (24,71) are the ones that are mainly involved in hepatobiliary transport and will be discussed in some detail. Other non-ABC transporters like P-type ATPases, e.g. the Cu-ATPase (73), also play a role. Until now, at least 8 ABC transporters have been shown to be expressed in the liver. However, because of progress in the field of genome sequencing and gene mapping, including the human genome project, more hepatic transporters will probably be identified in the near future.

3.3.1 The P-glycoproteins

The human multidrug resistance gene products MDR1 and MDR3 and their rat and mouse homologues mdr1a, mdr1b and mdr2 are members of the P-gp sub-family. These were the first ABC-transporters shown to be localized at the canalicular membrane (74,75). More recently, another member of this family, highly expressed in the liver, was cloned from pig and rat liver, and was called the sister of Pgp (Spgp) (76,77).

MDR1/mdr1 shows high expression in the apical domains of cells in colon, small intestine, liver and in the adrenals but also in the brain and in the placenta (78,79). This general body distribution points to a protective function towards potential toxic agents or, alternatively, to a role in the transport of ubiquitous endogenous compounds. The role of MDR1/ mdr1 in bile formation is not clear yet. Various physiological functions of MDR1/ mdr1 in the liver have been postulated, such as transport of endogenous and exogenous metabolites, steroid hormones, hydrophobic peptides or even glycolipids (24,78,80,81). In livers from xenobiotic treated animals, like carbontetrachloride, colchicine and α-naphtyl isothiocyanate, the expression of *mdr1* is increased (78). Studies in mdr1a/b knock-out mice revealed that absence of the gene product does not affect bile formation or composition. Currently mdr1 is thought to play a role in the removal of hydrophobic basic drugs and of more hydrophilic monoquartanary cations.

MDR3/mdr2 Pgp does not refer multidrug resistance to cells but is involved in the translocation of phospholipids across the canalicular membrane in an ATP-dependent fashion (82). This function became apparent when knock-out mice were developed in which the *mdr2* gene was disrupted (83). Absence of mdr2 Pgp leads to the complete absence of phospholipids in bile (83). It was shown that mdr2 Pgp acts as a flipase, that "flips" phosphatidyl choline molecules from the inner- to the outer leaflet of the membrane (79,82,84-86). It has been postulated that this induces the formation of PC-rich microdomains in the outerleaflet which are thought to be destabilized by bile salt micelles and, via an yet unknown mechanism, are able to release vesicular structures into the bile (84,87). In mdr2 P-gp-deficient mice, the secretion of cholesterol is also reduced and, possibly due to secondary events, glutathione secretion is also defective. These mice show an age- and sex-dependent liver pathology, including bile duct proliferation, portal inflammation and increased levels of gamma glutamyl transpeptidase (γGT), thought to be released from membranes by detergent activity of bile salts in the absence of protective phospholipids (83,88). Yet, these mice are not cholestatic since bile flow is actually increased relative to control mice. Recently, human homologues of this knockout mouse have been found that lack functional MDR3 in the liver (85,89,90). These patients were found to have a liver pathology similar to that seen in the mice and presented with increased cholestatic serum markers and high γGT levels (89,90). This human disease is now referred to as progressive familial intrahepatic cholestasis subtype 3 (PFIC-3) (90).

The third Pgp with high expression in the liver is the SPGP (76). In rat liver, *mdr2* and *spgp* mRNA levels are approximately equivalent and more abundant than those of the *mdr1a/b* genes (91). Therefore, spgp is thought to have an important function in hepatobiliary transport. Very recent observations provide strong evidence that spgp functions as a canalicular bile salt transporter for monovalent bile salts (92): expression of spgp in oocytes and subsequent kinetic studies of bile salt transport revealed similar Km and Vmax values for taurocholate transport as obtained in earlier studies in isolated rat membrane vesicles (92).

3.3.2 The Multidrug Resistance Proteins (MRP)

MRP1/ mrp1 was the first transporter of the mrp sub-family to be cloned. Originally, it was cloned from a small lung cancer cell line (93), but it was found to be expressed also in the liver (93,94). However, in normal hepatocytes the expression of mrp1 is low. In contrast, mrp1 is highly expressed in both HepG2 cells and in SV40 immortalized human hepatocytes (95). Furthermore, mrp1 is upregulated in proliferating hepatocytes (95,96). Mrp1 was found to be localized in the lateral membranes of adjacent cells (91,95). By using an MRP1 fragment as a probe, a second mrp, mrp2, was cloned from rat liver (97). Sequence analysis revealed that mrp2 has high homology with the human MRP1 (98,99). These transporters have a similar substrate specificity for a broad range of organic anions (94,100-102). In contrast to mrp1, mrp2 has a high expression in the liver, is localized in the canalicular membrane and was found to be absent in the GY/ TR$^-$ Wistar rat strain, identifying mrp2 as " the" canalicular multiple organic anion transporter formerly known as cmoat. Analysis of the mutation in GY/TR$^-$ rats revealed a 1 base pair deletion at amino acid 393 which results in a frame shift and a subsequent stop codon. As a consequence a truncated nonviable protein is produced that is readily broken down. Because of this premature stop in translation, the mRNA is also degraded faster, leading to low mRNA levels in these rats (97,98). Due to the absence of mrp2, these rats, like their counterparts the Corriedale sheep and humans with Dubin Johnson syndrome, have conjugated hyperbilirubinemia caused by a decreased potential to secrete bilirubin glucuronides. In addition to the mutation in GY/TR$^-$ rats, the mutation in Dubin Johnson patients has also been revealed recently (103-105). Studies in GY/TR$^-$ rats have learned much about the substrate specificity of this transporter, before it was actually cloned (Table). These studies were based on a reduced secretion of compounds considered to be potential substrates for the canalicular organic anion transporter. Interestingly, however, the secretion of some organic anions is more impaired than that of others, suggesting that for some substrates alternative transporters are available. These may be other members of the mrp family. By screening databases of human expressed sequence tags three new homologues of MRP1 and MRP2 have been identified very recently, MRP3, MRP4 and MRP5, of which only MRP3 is expressed mainly in the liver (99).

substrate	*negative charges*	*reference*
Endogenous compounds		
conjugated bilirubin	2	(115)
glutathione GSH	2	(7)
glutathione GSSG	4	(7)
cysteinyl-leukotrienes	2/3	(129)
triiodothyronine-glucuronide	2	(130)
coproporphyrin	2	(116)
Bile acid conjugates		
cholate 3-O-glucuronide	2	(131)
lithocholate 3-O-glucuronide	2	(131)
nordeoxycholate 3-O-glucuronide	2	(132)
tauro/ glyclithocholate 3-sulfate	2	(6)
taurochenodeoxycholate 3-sulfate	2	(6)
nordeoxycholate 3-sulfate	2	(132)
Exogenous compounds		
ceftriaxone	2	(133)
ampicillin	2	(134)
carboxyfluorescin	2	(135)
dibromosulfophtalein	2	(115)
bromosulfophtalein-glutathione	4	(115)
dinitrophenyl-glutathione	2	(7)
gluthationyl-bromoisovalerylurea	2	(136)
naphtol-1-glucuronide	1	(137)
Amino acids		
threonine	1	(7)
glutamate	2	(7)
isoleucine	1	(7)
leucine	1	(7)
tyrosine	1	(7)
phenylalanine	1	(7)
Metals		
zinc	-	(138)
copper*	-	(138)
manganese	-	(139)

**only exogenous, probably GSH complex*

Table 1. Substrates of the organic anion transporter mrp2 (78)

OUTLINE OF THE THESIS

A disturbance in the secretion or drainage of bile leads to a series of biochemical and / or morphological changes in the liver and, depending on its severity, to the accumulation of biliary components in the blood compartment. This combination of events is referred to as cholestasis (106-110). Cholestasis is functionally defined as an impairment or cessation of bile flow. Clearly, this definition is in practical terms only applicable in an experimental setting. In the clinical situation, cholestasis is usually characterized on the consequences of reduced bile secretion, i.e. accumulation of bilirubin, bile salts and on elevated liver enzymes in serum of patients. Due to the accumulation of conjugated bilirubin in serum, it generally referred to as jaundice. The processes underlying cholestasis can be extremely varied. Disturbance of bile flow can have an extrahepatic origin, for example obstruction of the bile ducts by tumors or gallstones, or it can develop at the level of the hepatocyte. In this case it is referred to as intrahepatic cholestasis. Intrahepatic cholestasis, in turn, can be caused by disturbances of any of the complex sequence of events involved in bile formation. Changes in membrane composition leading to altered fluidity of membrane structures can affect activity of the integral membrane-bound transporters. Reduced levels of these transporting proteins, as caused by decreased gene transcription and/or posttranscriptional events, may diminish overall transport capacity. Increased permeability of tight junctions may lead to regurgitation of bile components back to blood. In addition to processes at the level of the plasma membrane, processes inside the cell also can be involved, for example changes in intracellular transport processes, like microtubili derangement, or changes in the intracellular signaling cascade. When bile salt synthesis from cholesterol is affected, this may influence the magnitude of the bile salt-dependent fraction of bile flow. Thirdly, processes inside the canaliculi can underlie the development of cholestasis. It has been shown that substances like lithocholate and indocyanine green cause cholestasis after their secretion into bile by the formation of precipitates (111) that cause an obstruction of the canaliculi.

In the daily clinical practice, it is often very difficult to diagnose the cause of cholestasis in a jaundiced patient. Differential diagnosis based on combinations of serum parameters, sometimes enforced by specific liver function tests and/or genetic analysis may give clues on the primary events. Even then, the picture may become clouded by secondary effects caused by the presence of disturbances in bile formation in itself. Animal studies, in particular in bile duct ligated animals, have shown that a series of adaptive responses take place in the cholestatic liver, responses that may aggravate the "clinical picture". It should be realized, therefore, that deviations in biochemical parameters in blood do not always adequately reflect changes in bile formation.

In this thesis the relationship between transporter activity, bile formation and cholestatic serum parameters like bilirubin, bile salt and liver enzymes will be discussed using different experimental models. Specifically studies are focused on the basolateral bile salt uptake system ntcp, a protein that was cloned in the time that these studies started. Although the canalicular secretion of bile salts is the

rate limiting step in biliary secretion of bile salts, a relationship between cholestatic serum markers and down regulation of ntcp was suggested. In the work described in this thesis we relate changes in ntcp expression to actual bile formation. During the course of this study also the canalicular transporters mrp2, involved in organic anion transport, and mdr2-p-Glycoprotein, involved in canalicular lipid secretion were cloned and allowed us to include these in our studies. This approach allows to integrate the clinical view on cholestasis, which is primarily based on serum biochemistry into a more complete picture of bile formation and hepatobiliary transport in defined models of cholestatic liver disease.

METHODS

1. Animal models

Bile fistula rats. Evaluation of bile formation under unrestrained conditions is possible using rats equipped with permanent catheters in bile duct and duodenum (112). These catheters can be connected in order to maintain an intact enterohepatic circulation. Bile flow and composition can be studied under physiological conditions. When the enterohepatic circulation is interrupted by disconnecting both catheters, bile salt pool is depleted and hepatic bile salt synthesis can be measured directly by assessment of biliary bile salt output

GY/TR⁻ rat. The mutant GY/TR⁻ rat carries an autosomal recessive inherited defect in the canalicular organic anion transporter mrp2 that causes conjugated hyperbilirubinemia (7). This defect in rats resembles the one found in humans with Dubin Johnson syndrome (103-105). These mutants have a defective secretion of a great range of organic anions including bilirubin glucuronides (101), oxidized and reduced glutathione (113), glutathione-conjugates (7,102,113), cysteinyl-leukotrienes (7), DBSP, indocyanine green (7,114,115) and glucuronidated and sulfated bile salts (6). The secretion of bile salts like taurocholate is not affected, indicating that bile salts are not transported via this organic anion transporter (116). BSIDF is decreased by 50 % in these rats (116). This is thought to be caused, at least in part, by the diminished transport of reduced glutathione. During the course of the work described in this thesis, the gene encoding this transporter was cloned. These rats were shown to lack the mrp2 protein. Analysis of the mutation revealed a 1 base pair deletion at amino acid 393 which results in a frame shift and a subsequent premature stop codon (97). With this knowledge, this mutant rat has become a fully defined model to study the role of mrp2 in bile formation and cholestasis.

Mdr2 knock out mice. The final answer about the physiological function of mdr2 was provided when knockout mice were produced with a disrupted *mdr2* gene (83). These mice turned out to develop liver disease that is caused by the inability to secrete phospholipid into bile. Mice homozygous for the disrupted allele produce phospholipid- and cholesterol- depleted bile, while bile salt secretion is not

affected. The secretion of glutathione is also reduced in these mice. The mice, however, are not cholestatic taken into account their bile production, but do show a "cholestatic" serum. It is now clear that that mdr2 Pgp acts as a phospholipid flippase. Transfer of phosphatidylcholine from the inner to the outer leaflet of the membrane was shown in transfection studies in yeast (84), in fibroblast from transgenic mice that express the human homologue of mdr2, MDR3 (117), and in canalicular membranes isolated from rat liver (88). Recently, human homologues of this knockout mouse have been found that lack functional MDR3 in the liver (85,89,90). These patients were found to have a liver pathology similar to that seen in the mice and presented with cholestasis and high γGT levels (89,90). This human disease is now referred to as progressive familial intrahepatic cholestasis subtype 3 (PFIC-3) (90). Mdr2 Pgp knockout mice can be used for studying the role of biliary lipid secretion in the physiology as a whole, but also can provide information about its function in the regulation of other hepatic transporters.

Ferrochelatase-deficient mice. Another mutant mouse that can be used as a model for human hepatobiliary disease is the ferrochelatase-deficient mouse. This mouse reflects Erythropoetic Protoporphyria (EPP) in humans. Ferrochelatase is the last enzyme in the heme biosynthetic pathway and catalyzes the insertion of reduced iron (Fe^{2+}) into protoporphyrin. Reduced activity of this enzyme in humans leads to cutanous photosensitivity and accumulation of protoporphyrins in erythrocytes and liver and causes irreversible liver damage in the experimental mouse model and some patients. The mutation in the mice has been induced in a chemical mutagenesis experiment with ethylnitrosourea (118). This condition is transmitted in an autosomal recessive fashion. Molecular analysis of the defect revealed a T to A transversion at nucleotide 293, leading to a methionine to lysine substitution at position 98 of the protein and hence a defective enzyme (119). Interestingly, these mice show severe hyperbilirubenemia and portal fibrosis, indicating that these mice provide a model for studying the relations between liver disease, serum parameters of cholestasis transport activities and bile formation.

Ethinylestradiol treated rats. A widely used experimental model for intrahepatic cholestasis is the ethinylestradiol (EE)-treated rat (120). Administration of this synthetic estrogen in a relative high dose to rats causes a reduction of bile flow. This model has been advocated to resemble intrahepatic cholestasis of pregnancy (121). EE has profound inhibitory effect on the magnitude of the BSIDF (122,123), affects the expression and activity of a number of hepatic systems involved in bile formation (40,68,124) and alters hepatic plasma membrane fluidity (125). However, the exact mechanism underlying the reduction of bile flow in this model has not yet been defined.

2. In vitro techniques

In vitro transport. In addition to the animal models used in our studies, we also applied *in vitro* techniques for the study of hepatic transport. By specific isolation procedures basolateral and

canalicular membranes can be separated from each other and used for studying the transport kinetics of specific substrates, without the interference of other cellular processes (126-128). These procedures were available for rat, human and mice. We have adapted these procedures for the isolation of plasma membranes from livers of different pathological mouse models.

REFERENCES

1. Tavaloni N. The intrahepatic biliary epithelium: an area of growing interest in hepatology. Sem Liv Dis 1987;7:280-292.
2. Lamers WH, Hilberts A, Furt E, Smith J, Jonges GN, van Noorden CJF, Gaasbeek Janzen J, W., Charles R, Moorman AFM. hepatic enzymatic zonation: A reevaluationof the concept of the liver acinus. Hepatology 1989;10:72-76.
3. Quistorff B, Romert P. High-zone selectivity of cell permeabilization following digitonin-pulse-perfusion of rat liver: A reinterpretation of microcirculatory zones. Histochem 1989;92:487-498.
4. Ballatori N, Truong A, T. Relation between the biliary glutathione excretion and bile acid-independent bile flow. Am J Physiol 1989;256:G22-G30.
5. Ballatori N, Truong A, T. Glutathione as a primary osmotic driving force in hepatic bile formation. Am J Physiol 1992;263:G617-G624.
6. Kuipers F, Enserink M, Havinga R, van der Steen A, B, Hardonk MJ, Fevery J, Vonk RJ. Seperate transport systems for biliary secretion of sulfated and unsulfated bile acids in the rat. J Clin Invest 1988;81:1593-1599.
7. Oude Elferink RPJ, Ottenhoff R, Liefting W, de Haan J, Jansen PLM. Hepatobiliary transport of glutathione and glutathione conjugate in rats with hereditary hyperbilirubinemia. J Clin Invest 1989;84:476-483.
8. Hardison WGM, Wood CA. Importance of bicarbonate in bile salt-independent fraction of bile flow. Am J Physiol 1978;235:E158-E164.
9. Carey MC, Duane WC. Enterohepatic Circulation. In: Arias I, Fausto N, Boyer J, Jakoby W, Schachter D, Shafritz D, eds. The liver biology and pathobiology. New York: Raven Press, 1994:719-796.
10. Twisk J, Hoekman MFM, Mager WH, Moorman AFM, de Boer PAJ, Sheja L, Princen HMG, Gebhardt R. Heterogeneous expression of cholesterol 7a-hydroxylase and sterol 27-hydroxylase genes in the rat liver lobulus. J Clin Invest 1995;95:1235-1243.
11. Vlahcevic ZR, Heuman DM, Hylemon PB. Regulation of bile acid synthesis. Hepatology 1993;13:590-600.
12. Princen HMG, Post SM, Twisk J. Regulation of bile acid biosynthesis. Curr Pharm Design 1997;3:59-84.
13. Vlahcevic ZR, Pandak WM, Heuman DM, Hylemon PB. Function and regulation of hydroxylases involved in bile acid biosynthesis pathways. Sem Liv Dis 1992;12:403-419.
14. Russel DW, Setchell KDR. Bile acid biosynthesis. Biochemistry 1992;31:4737-4749.
15. Danielson H. Nair PP, Kritchevski D, eds. The bile acids. New York: Plenum Pres, 1973: 1-32.
16. Rodrigues CMP, Kren BT, Steer CJ, Setchell KDR. Formation of d 22-bile acids in rats is not gender specific and occurs in the peroxisome. J Lipid Res 1996;37:540-550.
17. Hofmann AF. Chemistry and Enterohepatic Circulation of Bile Acids. Hepatology 1984;4, No. 5:4S-14S.
18. Hofmann AF. Current concepts of biliary secretion. Dig Dis Sci 1998;34:16S-20S.
19. Smit MJ, Temmerman AM, Havinga R, Kuipers F, Vonk RJ. Short- and long-term effects of biliary drainage on hepatic cholesterol metabolism in the rat. Biochem J 1990;269:781-788.
20. Singer II, Kawa DW, Kazaziz DM, Alberts AW, Chen JS, Huff JW, Ness GC. Hydroxymethyl glutaryl-CoA reductase containing hepatocytes are distributed periportally in normal and mevinoline treated rat livers. Proc Natl Acad Sci 1984;81:5556-5560.
21. Li AC, Tanaka RD, Callaway K, Fogelman AM, Edwards PA. Localization of 3-hydroxy-3-methylglutaryl CoA synthase in the rat liver and intestine is affected by cholesteramine and mevinoline. J Lipid Res 1988;29:781-786.
22. Ugele B, Kempen HJM, Gebhardt R, Meijer P, Burger HJ, Princen HMG. Heterogeneity of rat liver parenchyma in cholesterol 7a-hydroxylase and bile acid synthesis. Biochem J 1991;276:73-77.
23. Meier PJ. Transport Polarity of Hepatocytes. Sem Liv Dis 1988;8. No.4:293-307.
24. Müller M, Jansen PLM. Molecular aspects of hepatobiliary transport. Am J Physiol 1997;35:G1285-G1303.
25. Meier PJ. Molecular mechanisms of hepatic bile salt transport from sinusoidal blood into bile. Am J Physiol 1995;269:G801-G812.
26. Meier PJ, Eckhardt U, Schroeder A, Hagenbuch B, Stieger B. Substrate specificity of sinusoidal bile acid and organic anion uptake systems in rat and human liver. Hepatology 1997;26:1667-1677.
27. Sweadner KJ, Goldlin SM. Active transport of sodium and potassium ions. Mechanism, function and regulation. Engl J Med 1980;302:777-783.
28. Hagenbuch B, Lübbert H, Stieger B, Meier PJ. Expression of the hepatocyte Na+/bile acid cotransporter in *Xenopus Leavis* oocytes. J Biol Chem 199;256:5357-5360.
29. Hagenbuch B, Stieger B, Foguet M, Lübbert H, Meier PJ. Functional expression cloning and characterization of the hepatocyte Na+/ bile acid cotransport system. Proc Natl Acad Sci 1991;88:10629-10633.
30. Hagenbuch B, Meier PJ. Molecular cloning, chromosomal localization, and functional characterization of a human liver Na+/ bile acid cotransporter. J Clin Invest 1994;93:1326-1331.

31. Stieger B, Hagenbuch B, Landmann L, Höchli M, Schroeder A, Meier PJ. In situ localization of the hepatocytic Na+/ Taurocholate cotransporting polypeptide in rat liver. Gastroenterology 1994;107:1781-1787.
32. Liang D, Hagenbuch B, Stieger B, Meier PJ. Parallel decrease of Na^+-taurocholate cotransport and its encoding mRNA in primary cultures of rat hepatocytes. Hepatology 1993;18:1162-1166.
33. Boyer JL, Ananthanarayanan M, Hofmann AF, Schteingart CD, Hagenbuch B, Stieger B, Meier PJ. Expression and characterization of a functional rat liver Na^+ bile acid cotransport system in COS-7 cells. Am J Physiol 1994;266:G382-G387.
34. Green RM, Lipin AL, Pelletier EM, Hagenbuch B, Meier PJ, Beier DR, Gollan JL. Hepatic regeneration is associated with a marked reduction in mRNA expression of the basolateral Na^+-taurocholate transporter. Gastroenterology 1994;106:A901(Abstract).
35. Green RM, Gollan JL, Hagenbuch B, Meier PJ, Beier DR. Regulation of hepatocyte bile salt transporters during hepatic regeneration. Am J Physiol 1997;273:G261-G267.
36. Bolder U, Ton-Nu HT, Schteingart CD, Frick E, Hofmann AF. Hepatocyte transport of bile acids and organic anions in endotoxemic rats: impaired uptake and secretion. Gastroenterology 1997;112:214-224.
37. Green RM, Beier DR, Gollan JL. Regulation of hepatocyte bile salt transporters by endotoxin and inflamatory cytokines in rodents. Gastroenterology 1996;111:193-198.
38. Moseley RH, Wang W, Takeda H, Lown K, Shick L, Ananthanarayanan M, Suchy FJ. Effect of endotoxin on bile transport in rat liver: a potential model for sepsis-associated cholestasis. Am J Physiol 1996;271:G137-G146.
39. Gartung C, Ananthanarayanan M, Rahman M, Schuele S, Nundy S, Soroka CL, Stolz A, Suchy FJ, Boyer JL. Down-regulation of expression and function of the rat liver Na^+/Bile acid cotransporter in extrahepatic cholestasis. Gastroenterology 1996;110:199-209.
40. Simon FR, Fortune J, Iwahashi M, Gartung C, Wolkoff AW, Sutherland E. Ethinyl estradiol cholestasis involves alterations in expression of liver sinusoidal transporters. Am J Physiol 1996;271:G1043-G1052.
41. Boyer JL, Hagenbuch B, Ananthanarayanan M, Suchy FJ, Stieger B, Meier PJ. Phylogenic and ontogenic expression of hepatocellular bile acid transport. Proc Natl Acad Sci 1993;90:435-438.
42. Karpen SJ, Sun AQ, Kudish B, Hagenbuch B, Meier PJ, Ananthanarayanan M, Suchy FJ. Multiple factors regulate the rat liver basolateral sodium dependent bile acid cotransporter gene promoter. J Biol Chem 1996;271:15211-15221.
43. Jaquemin E, Hagenbuch B, Stieger B, Wolkoff AW, Meier PJ. Expression cloning of rat liver Na+- independent organic anion transporter. Proc Natl Acad Sci 1994;91:133-137.
44. Kullack-Ublick GA, Hagenbuch B, Stieger B, Wolkoff AW, Meier PJ. Functional characterization of basolateral rat liver organic anion transporting polypeptide. Hepatology 1994;20:411-416.
45. Noe B, Hagenbuch B, Stieger B, Meier PJ. Isolation of multispecific organic anion and cardiac glycoside transporter from rat brain. Proc Natl Acad Sci 1997;94:10346-10350.
46. Grundemann d, Gorboulev V, Gamabaryan S, Veyhl M, Koepsell H. Drug excretion mediated by a new prototype of polyspecific transporter. Nature 1997;372:549-552.
47. Crawford JM, Berken CA, Gollan JL. Role of the hepatocyte microtubular system in the excretion of bile salts and biliary lipid: Implications for intracellular vesicular transport. J Lipid Res 1988;29:144-156.
48. Crawford JM, Strahs DCJ, Crawford AR, Barnes S. Role of bile salt hydrophobicity in the secretion of microtubule dependent bile salt secretion. J Lipid Res 1994;35:1738-1748.
49. Stolz A, Takikawa H, Sugiyama Y, Kuhlenkamp J, Kaplowitz N. 3a-hydroxysteroid dehydrogenase activity of the y' bile acid binders in the rat cytosol. J Clin Invest 1987;79:427-434.
50. Stolz A, Hammond L, Lou H. cDNA cloning and expression of the human hepatic bile acid binding protein. A member of the monomeric reductase family. J Biol Chem 1993;268:852-860.
51. Stolz A, Takikawa H, Ookthens M, Kaplowitz N. The role of cytoplasmatic proteins in hepatic bile acid transport. Ann Rev Med 1989;51:161-176.
52. Takikawa H, Stolz A, Kaplowitz N. Cyclical oxidation reduction of the C3 position on bile acids catalyzed by rat hepatic 3a-hydroxysteroid dehydrogenase. I. Studies with the purified enzyme, isolated rat hepatocytes, and inhibition by indomethacin. J Clin Invest 1987;80:852-860.
53. Takikawa H, Fernandez-Checa JCH, Kuhlenkamp J. Effect of indomethacin on the uptake, metabolism and excretion of 3-oxocholic acid: Studies in isolated hepatocytes and perfused rat liver. Biochim Biophys Acta 1991;1084:247-250.
54. Nathanson MH, Boyer JL. Mechanisms and regulation of bile secretion. Hepatology 1991;14 No.3:551-566.
55. Tanaka A, Katagiri K, Hoshino M, Hayakawa T, Tsukada K, Takeuchi T. Endothelin-1 stimulates bile acid secretion and vesicular transport in the isolated perfused rat liver. Am J Physiol 1994;266:G324-G329.
56. Renston RH, Zsigmond G, Bernhoft RA, Burwen SJ, Jones AL. Vesicular transport of horseradish peroxidase during chronic bile duct obstruction in the rat. Hepatology 1983;3:673-680.

57. Dumont M, D'Hont C, Durand-Schneider AM, Legrand-Defretin V, Feldmann G, Erlinger S. Inhibition by colchicine of biliary secretion of dimaleate in the rat: Evidence for microtuble-dependent vesicular transport. Hepatology 1991;14:10-15.
58. El-Seaidy A, Mills CO, Elias E, Crawford JM. Lack of evidence for vesicle trafficking of fluorescent bile slats in rats hepatocyte couplets. Am J Physiol 1997;272:G298-G309.
59. Crawford JM. Role of vesicular-mediated transport pathways in hepatocellular bile secretion. Sem Liv Dis 1996;16:169-189.
60. Alves C, von Dippe P, Amoui M, Levy D. Bile acid transport into hepatocyte smooth endoplasmatic reticulum vesicles is mediated by microsomal epoxide hydrolyse, a membrane protein exhibiting two distinct topological orientations. J Biol Chem 1993;268:20148-20155.
61. Maurice M, Schell MJ, Lardeux B, Hubbard AL. Biosynthesis and intracellulartransport of a bile canalicular plasma membrane protein: studies in vivo and in the perfused rat liver. Hepatology 1994;19:648-655.
62. Bartles JR, Feracci HM, Stieger B, Hubbard AL. Biogeneis of rat hepatocyte plasma membrane in vivo: comparison of the pathways taken by apical and basolateral protein using subcellular fractionation. J Cell Biol 1987;105:1241-1251.
63. Hubbard AL, Stieger B. Biogenesis of endogenous plasma membaren proteins in epithelial cells. Ann Rev Med 1998;51:755-770.
64. Oude Elferink RPJ, Bakker CTM, Roelofsen H, Middelkoop E, Ottenhoff R, Heijn M, Jansen PLM. Accumulation of organic anion in intracellular vesicles of cultured rat hepatocytes is mediated by the canalicular multispecific anion transporter. Hepatology 1993;17:434-444.
65. Boyer JL, Soroka CL. Vesicle targetting to the apical domain regulates bile excretory function in isolated rat hepatocyte couplets. Gastroenterology 1995;109:1600-1611.
66. Gautam ANO, Strazzabosco M, Boyer JL. Quantative assesment of canalicular bile formation in isolated rat hepatocyte couplets using microscopic optical planametry. J Clin Invest 1989;109:656-673.
67. Stieger B, Meier PJ, Landmann L. Effect of obstructive cholestasis on membrane traffic and domain-specific expression of plasma membrane proteins in rat liver parenchymal cells. Hepatology 1993;20,No. 1.201-212.
68. Trauner M, Arrese M, Soroka CL, Ananthanarayanan M, Koeppel TA, Schlosser SF, Suchy FJ, Keppler D, Boyer JL. The rat canalicular conjugate export pump (mrp2) is down regulated in intrahepatic and obstructive cholestasis. Gastroenterology 1997;113:255-264.
69. Arias IM, Che M, Gatmaitan ZC, Leveille C, Nishida T, Pierre St M. The biology of the bile canaliculus,1993. Hepatology 1993;17:318-329.
70. Gatmaitan ZC, Arias IM. ATP-dependent transport systems in the canalicular membrane of the hepatocyte. Physiol Rev 1995;75:261-275.
71. Dean M, Allikmets R. Evolution of ATP-binding cassette transporter genes. Curr Opin Genet Dev 1995;5:779-785.
72. Vore M. Canalicular transport: discovery of ATP dependent mechanisms. Toxicol Appl Pharmacol 1993;118:2-7.
73. Dijkstra M, In't Veld G, van den Berg GJ, Müller M. Adenosine triphosphate-dependent copper transport in isolated rat liver membranes. J Clin Invest 1995;95:412-416.
74. Silverman JA, Schrenk D. Expression of the multidrug resistance genes in the liver. FASEB J 1997;11:308-313.
75. Meijer DFK, Smit JW, Müller M. Hepatobiliary elimination of cationic drugs: the role of P-glycoproteins and other ATP-dependent transporters. Adv Drug Deliv Rev 1997;25:159-200.
76. Childs S, Yeh RL, Georges E, Ling V. Identification of a sister gene to P-glycoprotein. Cancer Res 1995;55:2029-2034.
77. Childs S, Yeh RL, Hui D, Wang R, Ling V. Isolation and expression of the liver-specific sister gene og P-glycoprotein. Hepatology 1997;26:297A(Abstract).
78. Oude Elferink RPJ, Meijer DFK, Kuipers F, Jansen PLM, Groen AK, Groothuis GMM. Hepatobiliary transport of organic compounds: multispecific carriers, P-glycoproteins and vesicular pathways. Biochim Biophys Acta 1995;1241:215-268.
79. Nies AT, Gatmaitan ZC, Arias IM. ATP-dependent phosphatidylcholine translocation in rat liver canalicular plasma membrane vesicles. J Lipid Res 1996;37:1125-1136.
80. Germann UA. P-glycoprotein-A mediator of multidrug resistance in tumour cells. Eur J Cancer 1996;32A:927-944.
81. van Helvoort A, Smith AJ, Sprong H, Fritzsche I, Schinkel AH, Borst P, van Meer G. MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 p-glycoprotein specially translocates phosphatidylcholine. Cell 1996;87:507-517.
82. Oude Elferink RPJ, Ottenhoff R, van Wijland MJ, Smit JJM, Schinkel AH, Groen AK. Regulation of biliary lipid secretion by mdr2 P-glycoprotein in the mouse. J Clin Invest 1995;95:31-38.
83. Smit JJM, Schinkel AH, Oude Elferink RPJ, Groen AK, Wagenaar E, van Deemter L, Mol CAAM, Ottenhoff R, van der Lugt NMT, van Roon MA, van der Valk MA, Offerhaus GJA, Berns AJM, Borst P. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell 1993;75:451-462.

84. Oude Elferink RPJ. The role of mdr2 P-glycoprotein in biliary lipid secretion. Cross-talk between cancer research and biliary physiology. J Hepatology 1995;23:617-625.
85. Dunia I, Smit JJM, van der Valk MA, Bloemendal H, Borst P, Benet LZ. Human multidrug resistance 3-P-glycoprotein expression in transgenic mice induces lens membrane alterations leading to cataract. J Cell Biol 1996;132:701-716.
86. Müller P, Pomorski T, Porwoli S, Tauber R, Herrmann A. Transverse movement of spin-labeled phospholipids in the plasma membrane of a hepatocytic cell line (hepG2): implications for biliary lipid secretion. Hepatology 1996;24:1497-1503.
87. Ruetz S, Gros P. Enhancement of mdr2-mediated phosphatidylcholine translocation by the bile salt taurocholate. Implications for hepatic bile formation. J Biol Chem 1995;270:25388-25395.
88. van Nieuwkerk CMJ, Groen AK, Ottenhoff R, van Wijland MJ, van den Berg Weerman MA, Tytgat GN, Offerhaus GJA, Oude Elferink RPJ. The role of bile salt composition in liver pathology of mdr2 (-/-) mice: differences between males and females. J Hepatology 1997;26:138-145.
89. Jaquemin E, de Vree JML, Sturm E, Cresteil D, Bosma PJ, Aten J, Deleuze JF, Desrochers M, Burdelski M, Bernard O, Hadchouel M, Oude Elferink RPJ. Mutations in the MDR3 gene are resposible for a subtype of progressive familial intrahepatic cholestasis. (PFIC). Hepatology 1997;26:284A(Abstract).
90. de Vree JML, Jaquemin E, Sturm E, Cresteil D, Bosma PJ, Aten J, Deleuze JF, Desrochers M, Burdelski M, Bernard O, Oude Elferink RPJ, Hadchouel M. Mutations inthe MDR3 gen cause progressive familial intrahepatic cholestasis. Proc Natl Acad Sci 1998;95:282-287.
91. Müller M, Vos TA, Koopen NR, Roelofsen H, Kuipers F, Jansen PLM. Localization and regulation of a novel canalicular ABC-transporter. Hepatology 1996;24:368A(Abstract).
92. Bjorkhem I. Inborn errors of metabolism with conseqences for bile acid biosynthesis. Scand J Gastroenterol 1994;29:68-72.
93. Loe DW, Deeley RG, Cole SPC. Biology of the multidrug resistance-associated protein, MRP. Eur J Cancer 1996;32A:945-957.
94. Slapak CA, Martell RL, Terashima M, Levy SB. Increased efflux of vincristine, but not of daunorubicin, associated with the murine multidrug resistance protein (MRP). Biochem Pharmacol 1996;52:1569-1576.
95. Roelofsen H, Vos TA, Schippers IJ, Kuipers F, Koning H, Moshage H, Jansen PLM, Müller M. Increased levels of the multidrug resistance protein in lateral membranes of proliferating hepatocyte-derived cells. Gastroenterology 1997;272:16-22.
96. Roelofsen H, Havinga R, Jansen PLM, Müller M. Differential regulation of two glutathione-*S*-conjugate transporters mrp1 and mrp2 during the initial phase of cell proliferation in hepatocytes. Hepatology 1996;24:258A(Abstract).
97. Paulusma C, Bosma PJ, Zaman GJ, Bakker CTM, Otter M, Scheffer GL, Scheper RJ, Borst P, Oude Elferink RPJ. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. Science 1996;271:1126-1127.
98. Büchler M, König J, Brom M, Kartenbeck J, Spring H, Horie T, Keppler D. cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. J Biol Chem 1996;271:15091-15098.
99. Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJT, Juyn J, Baas F, Borst P. Analysis of expression of cMOAT (MRP2), MRP3, MRP4 and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1) in human cancer cell lines. Cancer Res 1997;57:3537-3547.
100. Leier I, Jedlitschky G, Bucholz U, Cole SPC, Deely RG, Keppler D. The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. J Biol Chem 1994;269:27807-27810.
101. Jedlitschky G, Leier I, Buchholz U, Hummel-Eisenbeiss J, Burchell B, Keppler D. ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2. Biochem J 1997;327:305-310.
102. Keppler D, Leier I, Jedlitschky G. Transport of glutathione conjugates and glucuronides by the multidrug resistance proteins MRP1 and MRP2. Biol Chem 1997;378:787-791.
103. Paulusma C, Kool M, Bosma PJ, Scheffer GL, Ter B, F., Scheper RJ, Tytgat GN, Borst P, Baas F, Oude Elferink RPJ. A mutation in the human canalicular multispecific organic anion transporter gene causes the Dubin Johnson syndrome. Hepatology 1997;25:1539-1542.
104. Kartenbeck J, Leuschner U, Mayer R, Keppler D. Absence of the canalicular isoform of the mrp gen-encoded conjugate export pump from the hepatocytes in Dubin -Johnson syndrome. Hepatology 1996;23:1061-1066.
105. Paulusma C, Oude Elferink RPJ. The canalicular multispecific organic anion transporter and conjugated hyperbilirubinemia in rat and man. J Mol Med 1997;75:420-428.
106. Reichen J, Simon FR. Cholestasis. In: Arias I, Boyer J, Fausto N, Jakoby W, Schachter D, Shafritz D, eds. The liver: Biolology and Pathobiology. 3rd Ed. New York: Raven Press,Ltd. 1994:1291-1326.

107. Oelberg D, Lester R. Cellular mechanisms of cholestasis. Ann Rev Med 1986;37:279-317.
108. Fallon MB, Anderson JM, Boyer JL. Intrahepatic cholestasis. In: Schiff l, Schiff E, Lippinger J, eds. Diseases of the Liver. 7th Ed. Philadelphia: .Company, 1993:343-361.
109. Phillips MJ, Poucell S, Oda M. Biology of Disease Mechanisms of Cholestasis. Lab Invest 1986;54 No.6,593-608.
110. Javitt NB. Cholestatic Liver Disease: Mechanisms, Diagnosis and Therapy. In: AnonymousAdv Int Med. Year Book Medical Publishers, Inc. 1980:147-168.
111. Kuipers F, Havinga R, Van der Meer R, Koopen NR, Vonk RJ. The relationship between hepatobiliary transport and cholestatic properties of lithocholic acid conjugates in rats. In: Fromm H, Leuschner U, eds. Cholestatic liver disease. Dortrecht: Kluwer Academic Publishers, 1994:100-102.
112. Kuipers F, Havinga R, Bosschieter H, Toorop GP, Hindriks FR, Vonk RJ. Enterohepatic circulation in the rat. Gastroenterology 1985;88:403-411.
113. Paulusma C, van Geer M, Heijn M, Evers R, Ottenhoff R, Oude Elferink RPJ. The canalicular multispecific organic anion transporter mediates transport of reduced glutathione. Hepatology 1997;26:292A(Abstract).
114. Oude Elferink RPJ, Ottenhoff R, Radominska A, Hofmann AF, Kuipers F, Jansen PLM. Inhibition of glutathione conjugate secretion from isolated rat hepatocytes by dipolar bile acids and other organic anions. Biochem J 1991;274:281-286.
115. Jansen PLM, Groothuis GMM, Peters W, H.M., Meijer DFK. Selective hepatobiliary transport of organic anions and neutral steroids in mutant rats with heriditary-conjugated hyperbilirubinemia. Hepatology 1987;7:71-76.
116. Jansen PLM, Peters W, H.M., Lamers WH. Heriditary chronic conjugated hyperbilirubinemia in mutant rats caused by defective hepatic anion transport. Hepatology 1985;5:573-579.
117. Smith AJ, Timmermans-Hereijgers JLPM, Roelofsen H, Wirtz KWA, van Blitterswijk WJ, Smit JJM, Schinkel AH, Borst P. The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. FEBS Lett 1994;263-266.
118. Tutois S, Montagutelli X, Da Silva V, Jouault H, Rouyer-Fessard P, Leroy-Viard K, Guenet JL, Beuzard Y, Deybach JC. Erythropoietic protoporpheria in the house mouse. J Clin Invest 1991;88:1730-1736.
119. Boulechfar S, Lamoril J, Montagutelli X, Guenet JL, Deybach JC, Nordmann Y, Dailey H, Grandchamp B, De Verneuil H. Ferrochelatase structural mutant (*Fech^{m1pas}*) in the house mouse. Genomics 1993;16:645-648.
120. Vore M. Estrogen cholestasis membranes, metabolites, or receptors? Gastroenterology 1987;93:643-649.
121. Kreek MJ. Female sex steroids and cholestasis. Sem Liv Dis 1987;7:8-13.
122. Gumucio JJ, Valdivieso VD. Studies on the mechanism of the ethinylestradiol impairment of bile flow and bile salt secretion in the rat. Gastroenterology 1971;61:339-334.
123. Bouchard G, Yousef IM, Tuchweber B. Decreased biliary glutathione content is responsible for the decline in bile salt-independent flow induced by ethinyl estradiol in rats. Tox Let 1994;74:221-233.
124. Bossard R, Stieger B, O'Neil B, Fricker G, Meier PJ. Ethinylestradiol treatment induces multiple canalicular membrane transport alterations in rat liver. J Clin Invest 1993;91:2714-2720.
125. Isaacson Y, van Thiel DH. Increased membrane fluidity and cholestasis: associated but not linked consequences of estrogen treatment. J Lab Clin Med 1988;112:663-666.
126. Meier PJ, Sztul ES, Reuben A, Boyer JL. Structural and functional polarity of canalicular and basolateral plasma membrane vesicles isolated in high yield from rat liver. J Cell Biol 1984;98:991-1000.
127. Wolters H, Spiering M, Gerding A, Slooff MJH, Kuipers F, Hardonk MJ, Vonk RJ. Isolation and characterization of canalicular and basolateral plasma membrane fractions from human liver. Biochim Biophys Acta 1991;1069:61-69.
128. Emmelot P, Bos CJ, van Hoeven RP, van Blitterswijk WJ. Isolation of plasmamembranes from rat and mouse livers and hepatomas. In: Fleisher S, Packer L, eds. Methods in enzymology XXXI. New York, San Fransisco, London: Academic Press, 1974:75-90.
129. Huber M, Guhlmann A, Jansen PLM, Keppler D. Hereditary defect of hepatobiliary cysteinyl leukotriene elimination in mutants with defective hepatic anion excretion. Hepatology 1987;7:224-228.
130. de Herder WW, van der Hul RL, Bonthuis S, Jansen PLM, Oude Elferink RPJ, Visser TJ. Pathways for the biliary excretion of T3 conjugates. Ann Endocrin 1989;50:98-99.
131. Kuipers F, Radominska A, Zimniak P, Little JM, Havinga R, Vonk RJ, Lester R. Defective secretion of bile acid-3-O-glucuronides in rats with heriditary conjugated hyperbilirubinemia. J Lipid Res 1989;30:1835-1843.
132. Oude Elferink RPJ, de Haan J, Lambert KJ, Hagey LR, Hofmann AF, Jansen PLM. Selective hepatobiliary transport of nordeoxycholate side chain conjugates in mutant rats with canalicular transport defect. Hepatology 1989;9:861-865.
133. Oude Elferink RPJ. Biliary secretion of antibiotics. The mechanism of ceftriaxone secretion. In: Tytgat GJN, Huibregtse K, eds. Bile and bile duct abnormalities. Patho physiology, diagnosis and management. Stuttgart: Thieme, 1989:9-14.

134. Verkade HJ, Wolbers MJ, Havinga R, Uges DRA, Vonk RJ, Kuipers F. The uncoupling of biliary lipid secretion from bile acid secretion by organic anions in the rat. Gastroenterology 1990;99:1485-1492.
135. Jansen PLM, van Klinken JW, van Gelder M, Ottenhoff R, Elferink RP. Preserved organic anion transport in mutant TR' rats with hepatobiliary secretion defect. Am J Physiol 1993;265:G445-G452.
16. Polhuis M, Kuipers F, Vonk RJ, Mulder GJ. Stereoselectivity of glutathione conjugation: blood elimination of a-bromoisovalerylurea enantiomers and biliary secretion of conjugates in unanesthesized normal and congenitally jaundiced rats. J Pharmacol Exp Ther 1989;249:874-878.
137. de Vries MH, Redegeld FAM, Koster AS, Noordhoek J, de Haan J, Oude Elferink RPJ, Jansen PLM. Hepatic, intestinal and renal transport of 1-naphtol-b-D-glucuronide in mutant rats with heriditary conjugated hyperbilirubinemia. Naunyn-Schmied Arch.Pharmacol. 1989;340:588-592.
138. Houwen RHJ, Dijkstra M, Kuipers F, Havinga R, Vonk RJ. Two pathways for biliary copper excretion in the rat. Biochem Pharmacol 1990;39:1039-1044.
139. Dijkstra M, Kuipers F, Havinga R, Smit EP, Vonk RJ. Bile secretion of trace elements in rats with congenital defect in hepatobiliary transport of glutathione. Pediatr Res 1990;28:339-343.

Chapter 2

Impaired activity of the bile canalicular organic anion transporter (mrp2/cmoat) is not the main cause of ethinylestradiol-induced cholestasis in the rat.

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ABSTRACT

To test the hypothesis that impaired activity of the bile canalicular organic anion transporting system mrp2 (cmoat) is a key event in the etiology of 17α-ethinylestradiol (EE)-induced intrahepatic cholestasis in rats, EE (5 mg/kg s.c. daily) was administered to male normal Wistar (NW) and mrp2-deficient GY/TR$^-$ rats. Elevated plasma bilirubin levels in GY/TR$^-$ rats increased upon EE-treatment from 65 ± 8.4 μM to 183 ± 22.7 μM within three days whereas bilirubin levels remained unaffected in NW rats. Biliary bilirubin secretion was 1.5-fold increased in NW rats but remained unaltered in GY/TR$^-$ rats. Plasma bile salt concentrations remained unchanged in both strains, although hepatic levels of the sinusoidal Na^+-taurocholate cotransporting protein (ntcp) were markedly reduced. Biliary secretion of endogenous bile salt was not affected in either strain. A clear reduction of mrp2 levels in liver plasma membranes of NW rats was found after 3 days of treatment. The bile salt-independent fraction of bile flow (BSIF) was reduced from 2.6 to 2.0 μl/min/100 g body weight in NW rats with a concomitant 62% reduction of biliary glutathione secretion. The absence of mrp2 and biliary glutathione in GY/TR$^-$ rats did not prevent induction of EE-cholestasis: a similar absolute reduction of BSIF, i.e., from 1.1 to 0.6 μl/min/100 g body weight, was found in these animals. EE-treatment caused a reduction of the maximal biliary secretory rate (S_{RM}) of the mrp2 substrate dibromosulphthalein from 1040 to 695 nmol/min/100g body weight (-38%) in NW rats and from 615 to 327 nmol/min/100g body weight (-46%) in GY/TR$^-$ rats. These results demonstrate that inhibition of mrp2 activity and/or biliary glutathione secretion is not the main cause of EE-induced cholestasis in rats. The data indicate that alternative pathways exist for the biliary secretion of bilirubin and related organic anions that are also affected by EE.

INTRODUCTION

The synthetic estrogen 17α-ethinylestradiol (EE) causes a reversible intrahepatic cholestasis in experimental animals, mainly by reducing the bile salt-independent fraction of bile flow (BSIF) (1). Although EE-induced cholestasis has been claimed to represent a model of cholestasis of pregnancy in humans (2), the mechanism(s) underlying the reduction in bile flow have not been defined. Suggestions that increased permeability of tight junctions (3,4) and reduced activity of Na^+/ K^+-ATPase at the sinusoidal membrane (5-7) represent primary events in the onset of cholestasis could not be substantiated in more recent studies (8-11). Likewise, the relevance of the reported decrease in sinusoidal membrane fluidity after EE-treatment remains questionable, since decreased membrane fluidity has also been found in experimental conditions associated with increased bile flow (11,12). More recently, it was shown that EE differentially affects the activity of transport systems involved in bile formation in the sinusoidal and bile canalicular domains of rat liver. Thus, Bossard *et al.* (13) showed that sinusoidal Na^+-dependent taurocholate transport is reduced by 40% in livers from EE-treated (5 mg/kg/day for 5 days) rats and that the maximal velocity of ATP-dependent taurocholate transport in canalicular membrane vesicles is reduced by 63%. Because EE mainly affects the BSIF (1), it is probably of greater importance that ATP-dependent transport of S-(2,4-dinitrophenyl) glutathione (DNP-SG) is also reduced in canalicular membrane vesicles isolated from livers of EE-treated rats (13). DNP-SG is a substrate for the canalicular multispecific organic anion transporter (cmoat), identified as the canalicular isoform of the multidrug resistance protein (mrp2) (14). Mrp2 is involved in hepatobiliary transport of a wide variety of endogenous and exogenous compounds (15). Very recently, Trauner *et al.* (16) reported decreased levels of mrp2 in canalicular membranes isolated from livers of EE-treated rats and concluded that this could be a reason for decreased organic anion transport in EE-cholestasis. Absence of cmoat/mrp2 in mutant rat strains like GY/TR$^-$ (17,18) and EHBR (19,20) leads to hyperbilirubinemia and diminished bile flow due to a reduction of the BSIF (17). This reduction of bile flow has been attributed to the absence of glutathione (GSH) in the bile of these animals (21). GSH is considered to be the main determinant of BSIF in rodents (22) and reduced biliary GSH secretion is an early feature of EE-cholestasis in rats (23). To test the hypothesis that impaired activity of mrp2 is a direct cause of EE-induced cholestasis in rats, we have compared the effects of EE administration on plasma markers of cholestasis and on bile formation and composition in normal (NW) rats and mutant GY/TR$^-$ rats.

MATERIALS AND METHODS

Materials. 17α-Ethinylestradiol (EE) was purchased from Sigma Chemicals (St. Louis, MO,USA). Radiolabeled EE, 17α-[6,7-^{3}H(n)]ethinylestradiol (46.8 μCi/mmol) was obtained from New England Nuclear (Boston, MA,USA). Dibromosulphthalein (DBSP) was obtained from Serb (Paris, France). A polyclonal mrp2 antibody was raised against the carboxy terminal part of rat mrp2 (24). The polyclonal ntcp antibody (K4) was a kind gift from dr. B. Stieger (Division of Clinical Pharmacology and Toxicology, Department of Medicine, University Hospital Zürich, Zürich, Switzerland). All other chemicals were of reagent grade or the highest purity grade commercially available.

Animals. Male Wistar and mutant GY/TR$^-$ Wistar rats, bred at the Central Animal Laboratory of the Faculty for Medical Sciences, University of Groningen, of 290-330 g were used for these studies. Animals were kept in a light- and temperature-controlled environment and had free access to lab chow and tapwater throughout the experiments. The animals received humane care and experimental protocols complied with the local guidelines for use of experimental animals.

EE treatment. Rats received subcutaneous injections of EE (5 mg/kg) or of the solvent, i.e., 1,2-propanediol, for three or five consecutive days, as indicated in the result section.

In vivo studies. Biliary and urinary secretion of radiolabeled EE (metabolites) was quantified in three NW rats and three GY/TR$^-$ rats with long-term bile diversion (25) kept in metabolic cages. For this purpose, rats received a single subcutaneous injection of 5 mg/kg of ^{3}H-EE diluted to a specific activity of 43 μCi/mmol with cold material. Bile was subsequently collected continuously for 48 hours as specified in the legend of figure 1. Urine was collected in two 24 h samples.
To study the effects of EE on plasma parameters, rats were equipped with a permanent catheter in the jugular vein (25). These rats were treated with EE for 5 consecutive days and blood samples were taken before treatment and at days 1, 3, and 5.
To study the effects of EE on bile formation and composition under unrestrained conditions, separate groups of rats were equipped with permanent catheters in bile duct and duodenum as described in detail elsewhere (25). Both catheters were immediately connected to each other to maintain an intact enterohepatic circulation. EE or solvent (1,2-propanediol) injections were started four days after surgery, i.e., after animals had regained their preoperative body weights. After three days of treatment, the connection between both catheters was interrupted and bile was collected for 6 hours in 30 min intervals by means of a fraction collector. Bile volume was determined gravimetrically and samples were immediately stored at -20 °C for later analysis. A small aliquot of bile was immediately diluted in 3.5 % perchloric acid for GSH determination.

The maximal secretory rate (S_{RM}) of dibromosulphthalein (DBSP) into bile was determined by primed-constant infusion of the dye (7.7 μmol followed by 23.1 μmol/h) in 3 days solvent- or EE-treated NW and GY/TR$^-$ bile-fistula rats (n = 3 per group) kept under pentobarbital anesthesia.

Liver plasma membrane isolation. Separate groups of solvent-treated and EE-treated NW and GY/TR$^-$ rats were used for isolation of hepatic plasma membranes by density gradient ultracentrifugation (26). The membrane aliquots were frozen and stored until use at -80°C.
Protein concentrations were determined according to Lowry (27). Relative enrichments of Na^+ / K^+-ATPase as marker enzyme for the basolateral fraction and Mg^{2+}-ATPase, leucine aminopeptidase and alkaline phosphatase as marker enzymes for the canalicular fraction, i.e. activity of the enzyme in the isolated plasma membrane preparation divided by the activity in the homogenate, was used to determine the degree of purification of the isolated membranes in the different experimental groups. Leucine aminopeptidase (28), Na^+/ K^+ ATPase and Mg^{2+}ATPase activity (29), alkaline phosphatase (30), glucose-6-phosphatase (31) and succinate cytochrome C reductase (32) were measured using a Uvikon 931 spectrophotometer (Kontron Instruments, Milan, Italy).

DPH-fluorescence depolarization. The total plasma membrane fraction (100μg protein) of each experimental group was used for DPH-fluorescence depolarization determination and measured in duplicate in three preparations according to Wolters *et al.* (33)

Western blotting. Approximately 15 μg of protein of total plasma membrane fraction of each experimental group, normalized for enrichment in Mg^{2+}ATPase or Na^+/ K^+ ATPase, was separated using SDS gel electrophoresis, transferred to nitrocellulose membrane (Amersham, Little Chalfont, UK), and probed with anti-mrp2- imunoglobulin(Ig)G and anti-ntcp-immunoglobulin(Ig)G K4, respectively. Immune complexes were detected using horseradish peroxidase-conjugated donkey anti rabbit IgG by the ECL Western blotting kit (Amersham, Little Chalfont, UK). Protein density was determined by scanning the blots using an Image Master VDS system (Pharmacia Biotech, Upsalla, Sweden)

Analyses. Bile salts in plasma and bile were determined by an enzymatic fluorimetric assay (34). Plasma cholesterol and triglycerides were measured enzymatically using commercially available kits (Boehringer Mannheim, Mannheim, Germany). Biliary cholesterol, phospholipids and GSH were measured exactly as previously described (25). Aspartate amino transferase (ASAT), alanine amino transferase (ALAT), bilirubin, amino acid concentrations in bile and biliary HCO_3^- were assessed by standard laboratory techniques.

Statistics. Data are expressed as mean ± SD for the indicated number of experiments. Data were analyzed using ANOVA, followed by the Student- Newman-Keuls test. Statistical significance was considered at P values < 0.05.

RESULTS

Disposition of radiolabeled EE. To assess the role of mrp2 in EE metabolism, we first determined the disposition of EE into bile and urine in rats with long-term bile diversion. Figure 1 shows that the amount of radioactivity secreted into bile after a single subcutaneous injection of ^{3}H-EE (5 mg/kg) was clearly reduced in GY/TR^{-} rats when compared to NW rats, indicating a role for mrp2 in the secretion process. Total recovery of radioactivity in bile amounted up to 33% ± 6.5 % dose in GY/TR^{-} rats and 88% ± 1.6 % dose in NW rats over a 48 h period. The GY/TR^{-} rats secreted a significantly larger part of the dose into the urine than NW rats did, i.e., 24.7 % ± 5.5 % vs. 1.4 % ± 0.2 % in 48 hours.

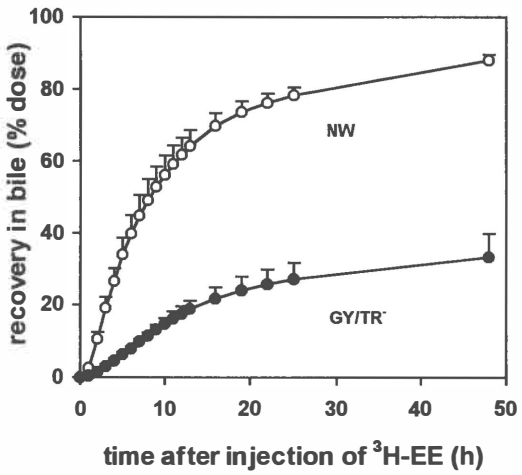

Figure 1. Disposition of ^{3}H-EE (metabolites) in bile of NW rats (open symbols) and GY/TR^{-} rats (closed symbols). *^{3}H-EE (5mg/kg) was injected subcutaneously into bile diverted NW and GY/TR^{-} rats. Bile was collected in 1 hr fractions during the first 13 hours of the experiment, followed by four 3 h-fractions and a single fraction of 24h. Radioactivity in bile was determined by scintillation counting. Results are expressed as cumulative % dose secreted into bile over time. Mean values ± SD are shown for 3 animals per group.*

Plasma parameters. Figure 2 shows that treatment of GY/TR^{-} rats with EE for up to 5 days resulted in a rapid increase in plasma bilirubin levels that reached its highest values after 3 days of treatment

(+280%). Plasma bilirubin levels remained unaffected in NW rats during treatment. As shown earlier (17), plasma bile salt concentrations are higher in GY/TR$^-$ rats than in NW rats; EE-treatment did not affect bile salt levels. Also the activities of the liver enzymes ASAT and ALAT in plasma did not alter. Treatment with EE led to significant decreases of plasma cholesterol levels, from 1.82 ± 0.06 to 0.09 ± 0.03 mM in NW rats and from 2.13 ± 0.20 to 0.24 ± 0.03 mM in GY/TR$^-$ rats, respectively, and of plasma triglyceride levels, from 0.84 ± 0.11 to 0.37 ± 0.02 mM in NW rats and from 0.73 ± 0.06 to 0.43 ± 0.01 mM in GY/TR$^-$ rats, respectively.

Liver (plasma membrane) composition. Administration of EE for three consecutive days resulted in a similar weight loss in NW (-2.4%) and in GY/TR$^-$ rats (-3.2%). The solvent treated groups did not lose bodyweight,+4.4% and +2.1%, for NW and GY/TR$^-$ rats, respectively. EE treatment of NW rats caused an increase in the liver weight/body weight ratio (table 1). As previously reported (17), livers of GY/TR$^-$ rats are significantly heavier than those of NW rats. The liver weight/body weight ratio in GY/TR$^-$ rats did not increase further upon EE treatment. Free cholesterol (NW: +24 % and GY/TR$^-$: +12 %) and cholesterylester contents (NW: +243 % and GY/TR$^-$: +138 %) were significantly increased in the EE-treated groups of both strains, as also reported for normal rats by others (35). Liver glutathione content was not affected by EE-treatment. No changes in liver morphology were observed in rats of either strain after EE-treatment upon microscopic evaluation of paraffin-embedded and frozen sections (data not shown).

	liver weight as % of body weight	*% change*
NW, control	4.08 ± 0.17	
NW, EE	4.88 ± 0.20 *	+ 20%
GY/TR$^-$, control	5.08 ± 0.12	
GY/TR$^-$, EE	5.10 ± 0.19	+ 1%

NOTE. Data are given as means ± SD (n=8)

** P<0.001*

Table 1. Effect of EE-treatment for 3 days on relative liver weight in normal Wistar (NW) and mutant (GY/TR$^-$) rats.

Table 2 summarizes enzyme activities measured in liver homogenates of solvent-treated and EE-treated NW and GY/TR$^-$ rats. The activities of the canalicular marker enzymes alkaline phosphatase and leucine aminopeptidase were significantly increased by EE-treatment in both strains, and Mg^{2+}/ATPase activity was increased in the EE-treated GY/TR$^-$ rats only. No significant effects on hepatic Na^+/K^+-ATPase activity were noted with this treatment schedule.Plasma membranes

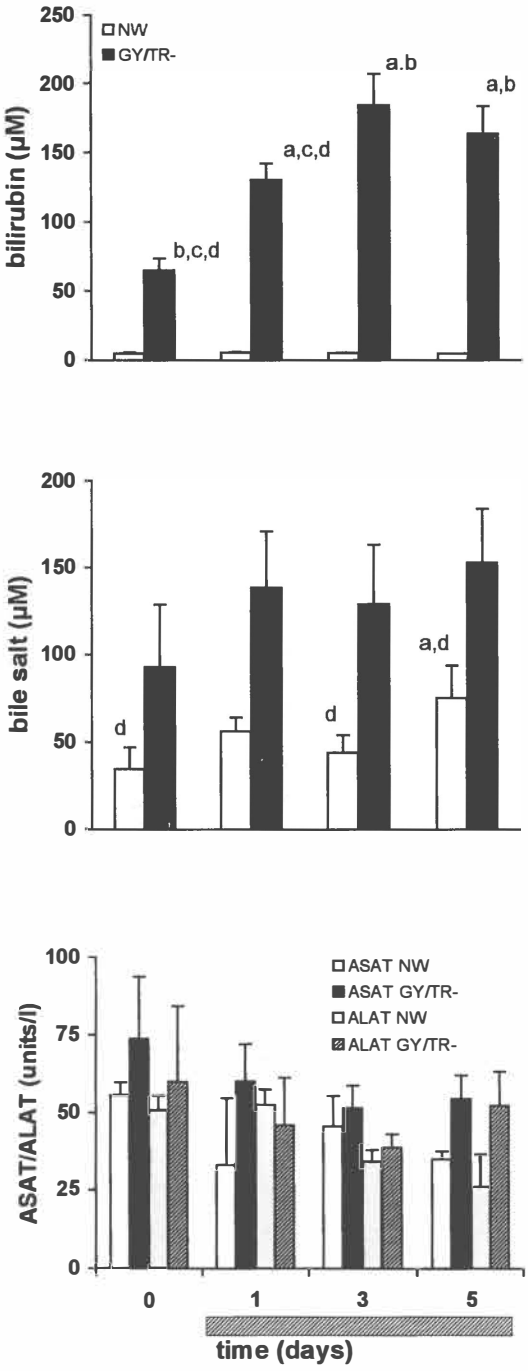

Figure 2. *Plasma total bilirubin concentration (top), bile salt concentration (middle) and ASAT/ ALAT activities (bottom) during 5 days of treatment with EE. Mean values ± SD are given for 5 animals per group; a, b, c and d indicate significant difference compared to day 0 (pretreatment values), day 1, day 3 and day 5, respectively.*

isolated from these livers were enriched in membrane marker enzyme activities to a similar extent across all groups (table 3). EE treatment had only modest effects on membrane phospholipid, cholesterol and cholesterylester content in both strains; only the phospholipid content of plasma membranes of the EE-treated NW rats was significantly reduced. Membrane fluidity, as assessed by DPH fluorescence depolarization was slightly decreased by EE-treatment in both groups, but the difference was only statistically significant in the EE-treated NW rats (table 4).

enzyme activity (*μmol/mg protein/hr*)	*NW, control*	*NW, EE*	*GY/TR⁻, control*	*GY/TR⁻, EE*
Na^+/K^+ATPase	0.92 ± 0.20	1.05 ± 0.31	1.01 ± 0.24	1.25 ± 0.25
Mg^{2+}ATPase	2.70 ± 0.63	3.29 ± 0.59	2.50 ± 0.18	3.10 ± 0.40 **
Leucine aminopeptidase	0.46 ± 0.04	0.58 ± 0.07 **	0.49 ± 0.05	0.58 ± 0.06 **
Alkaline phosphatase	0.38 ± 0.18	1.14 ± 0.49 **	0.27 ± 0.07	0.41 ± 0.13*
Succinate cyt. C reductase	4.92 ± 0.85	5.19 ± 0.90	5.50 ± 0.59	5.83 ± 0.76
Glucose-6-phosphatase	5.23 ± 0.80	4.65 ± 0.75	5.23 ± 1.35	4.98 ± 0.64

NOTE. Data are given as means ± SD (n=7)

** P< 0.05*

*** P<0.01*

Table 2. Effect of EE treatment for 3 days on enzyme activities in liver homogenates of normal Wistar (NW) and mutant (GY/TR⁻) rats.

enzyme	*NW, control*	*NW, EE*	*GY/TR⁻, control*	*GY/TR⁻, EE*
Na^+/K^+ATPase	25.2 ± 9.8	19.4 ± 3.3	31.2 ± 5.2	28.2 ± 3.0
Mg^{2+}ATPase	26.8 ± 4.9	39.6 ± 8.2	26.6 ± 3.1	30.8 ± 4.4
Leucine aminopeptidase	9.6 ± 2.0	11.3 ± 0.1	6.2 ± 0.2	6.5 ± 1.3
Alkaline phosphatase	22.9 ± 10.8	26.6 ± 3.5	9.2 ± 0.8	10.1 ± 0.8
Succinate cytochrome C reductase	1.24 ± 0.47	0.7 ± 0.09	1.09 ± 0.18	0.91 ± 0.12
Glucose-6-phosphatase	0.26 ± 0.05	0.22 ± 0.04	0.28 ± 0.07	0.32 ± 0.08

NOTE. Data are given as means ± SD of the relative enrichments (ratio of specific activities of the plasma membranes relative to those in total homogenates) for n=3 per group.

Table 3. Characterization of plasma membranes isolated from normal Wistar (NW) and mutant (GY/TR⁻) rats with and without EE treatment; marker enzyme enrichments

	NW, control	*NW, EE*	*GY/TR⁻, control*	*GY/TR⁻, EE*
phospholipids	431 ± 16	367 ± 9 *	387 ± 25	388 ± 14
cholesterol	220 ± 24	190 ± 11	196 ± 21	191 ± 25
cholesterylesters	45 ± 14	28 ± 9	23 ± 6	32 ± 7
P_{DPH}	0.270 ± 0.003	0.288 ± 0.005 **	0.277 ± 0.001	0.283 ± 0.006

NOTE. Phospholipids, cholesterol and cholesterylesters content in nmol/mg protein, as means ± SD for n=3 plasma membrane preparations per group.
** P<0.05*
*** P<0.01*

Table 4. Effect of EE-treatment for 3 days on lipid composition and DPH fluorescence depolarization of plasma membranes in normal Wistar (NW) and mutant (GY/TR⁻) rats.

Mrp2 and ntcp content of hepatic plasma membranes. Figure 3a shows representative Western blots of liver plasma membrane fractions from solvent-treated and EE-treated NW rats stained for mrp2. The amounts of protein applied to the gel were normalized to the enrichment of the canalicular marker enzyme Mg^{2+}-ATPase to allow comparison between the groups. The amount of mrp2 protein was reduced by 35% upon EE treatment as determined by densitometric scanning of the Western blots. As reported by Paulusma et al. (14), mrp2 could not be detected in GY/TR⁻ membranes. For ntcp protein assessment (Figure 3b), the amounts of protein applied to the gel were normalized to the enrichment of the sinusoidal marker enzyme Na^+/K^+ -ATPase to allow comparison between the groups. EE-treatment caused decreased ntcp levels in NW and GY/TR⁻ rats; this EE effect appeared to be more prominent in GY/TR⁻ rats. As shown earlier (36), ntcp appeared as a characteristic doublet upon Western blotting, with one band at 51 kD and another, minor, band at 56 kD. The latter band was affected most by EE treatment, as recently also shown by Simon *et al.* (37).

Bile formation. The effects of EE-treatment for three days on bile flow and on bile secretion rates of bile salts, bilirubin, GSH and total amino acids are shown in figure 4. The presented data represent output rates during the first hour after interruption of the enterohepatic circulation of unanaesthesized rats and are thus considered to approach 'physiological values'. As expected, bile flow was reduced by EE in NW rats, from 7.16 ± 0.73 to 5.55 ± 0.66 μl/min/100g body weight. A very similar reduction was observed in the mutants rats from 4.73 ± 0.43 to 3.59 ± 0.66 μl/min/100g body weight (panel A). Bile salt secretion was not affected in either strain (panel B). Bilirubin output in NW rats increased after EE-treatment, but remained unaltered in EE-treated

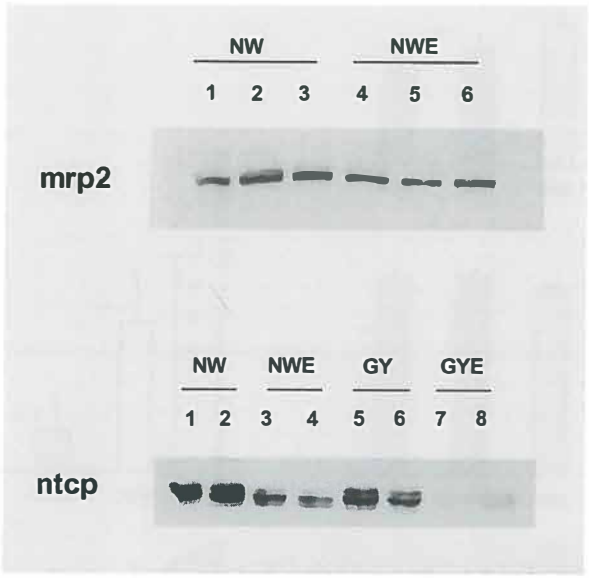

Figure 3. *Representative Western blots of mrp2 (a) and ntcp (b) on plasma membranes fractions of seperate isolations from control and EE-treated NW and GY/TR⁻ rats. NW and GY indicate solvent injected NW and GY/TR⁻ rats and NWE and GYE indicate EE-injected NW and GY/TR⁻ rats, respectively. Localization of molecular weight markers are indicated at the right hand side of the blots.*

TR⁻ rats (panel C). GSH output was below detection limits in GY/TR⁻ rats and was reduced from 9.65 ± 3.27 to 2.8 ± 2.05 μmol/min/100g body weight in NW rats by EE administration (panel D), as confirming earlier work by Bouchard *et al.* (23). Total amino acid secretion was lower in the GY/TR⁻ rats than in NW rats, but was unaffected by EE-treatment in both strains. Biliary secretion of glycine, a product of intracanalicular GSH hydrolysis, was reduced by about 50% in GY/TR⁻ rats in comparison to NW controls and remained unaltered after EE-treatment (data not shown). Biliary HCO_3^- concentrations were similar in all groups (about 55 mM). Therefore, HCO_3^- output rates varied according to the changes in bile flow.

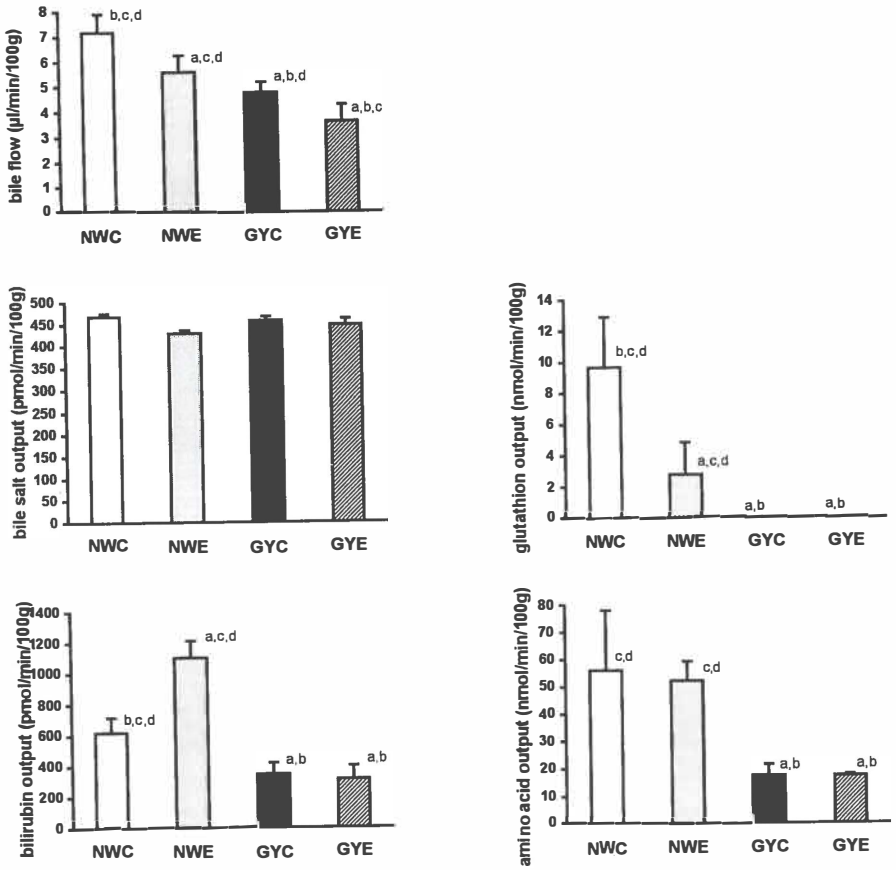

Figure 4. *Effects of EE treatment for three days on bile flow (a) and secretion rates of bile salts (b), bilirubin (c), glutathione (d) and amino acids (e), as determined in bile collected during the first hour after interruption of the enterohepatic circulation in NW and GY/TR rats. NWC and GYC indicate solvent injected NW and GY/TR rats and NWE and GYE indicate EE-injected NW and GY/TR rats, respectively. Mean values ±SD are given for 5-6 animals per group. a, b, c and d indicate significant difference compared to NWC, NWE, GYC and GYE, respectively*

Figure 5 shows the 'classical' relationship between bile salt output and bile flow in the four experimental groups. This figure was composed by plotting bile salt output rates determined in sequential 30 min intervals during 6 hours immediately after interruption of the enterohepatic circulation, i.e., during depletion of the intestinal bile salt pool (25), against simultaneously determined bile flow rates. This figure shows that EE-treatment induced a similar reduction of BSIF in both strains from 2.6 to 2.0 µl/min/100g body weight in NW rats and from 1.1 to 0.6 µl/min/100g body weight in GY/TR$^-$ rats. The choleretic potency of bile salts, indicated by the slope of the plotted relationship, was similar in NW and mutant rats, as reported earlier (17), and unaffected by EE administration.

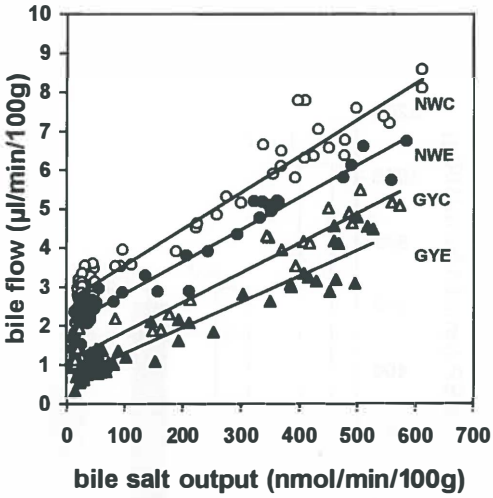

***Figure 5**. Relationship between bile flow and bile salt excretion in control and EE treated NW and GY/TR rats. Bile was collected from unanesthesized rats in 30 min fractions for 6 hours after interruption of the enterobepatic circulation, i.e., during depletion of the intestinal bile salt pool. Individual time points for 5-6 rats per group are shown. Regression analysis yielded equations of: NWC: y= 0.0094 x+2.62, GYC: y= 0.0076 x+1.07, NWE: y= 0.0083 x+1.99, GYE: y= 0.0067 x+0.63. Correlation coefficient was > 0.94 for all groups.*

Effects of EE on maximal secretory rate (S_{RM}) of dibromosulphthalein (DBSP). EE-treatment lowered the S_{RM} of DBSP in NW rats from 1040 ± 107 to 659 ± 34 ($p<0.05$) nmol/min/100 g body weight (Figure 6a). The S_{RM} of DBSP in GY/TR$^-$ rats was reduced from 615 ± 44 to 327 ± 23 nmol/min/100g body weight by EE-treatment. Regression analysis revealed a strong correlation between bile flow and S_{RM} of DBSP in these experiments, when data of the 4 experimental groups were pooled (Figure 6b).

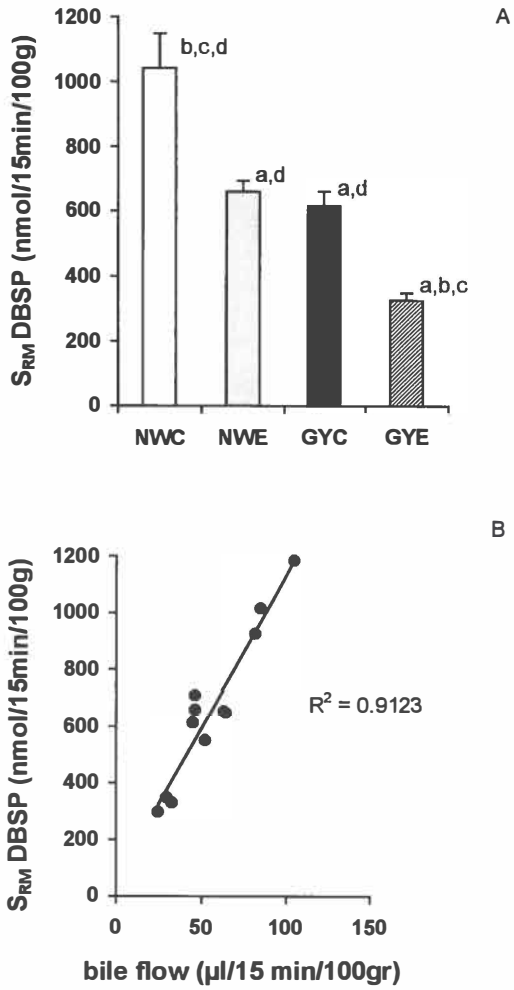

Figure 6 a. Transport maximum (S_{RM}) of DBSP in control and EE treated NW and GY/TR rats. *DBSP (7.7 µmol followed by 23.1 µmol/h) was administered to pentobarbital anestesized bile fistula rats. Bile was collected continuously for 90 min in 15 min fractions. DBSP output plateaued in all groups examined during the last period of the experiment. Mean values of 60-75 and 75-90 min were calculated to represent S_{RM}. Data are means ± SD for 3 animals per group: a, b, c and d indicate significant difference compared to NWC, NWE, GYC and GYE, respectively.*

Figure 6 b. Relation between transport maximum (S_{RM}) of DBSP and bile flow in control and EE treated NW and GY/TR rats. *Individual S_{RM} values for rats of the four experimental groups are shown.*

DISCUSSION

In order to evaluate the proposed role of mrp2 in the etiology of EE-induced cholestasis in rats (13,16), we compared the effects of EE-treatment in normal Wistar rats and mrp2-deficient GY/TR$^-$ rats. A single-nucleotide mutation in the *mrp2* gene leads to the absence of the mrp2 protein in the liver of GY/TR$^-$ rats (14) and, as a consequence, to reduced hepatobiliary transport of a variety of organic anions including GSH (18,38). This rat strain therefore is an unique model to evaluate the involvement of mrp2 and biliary GSH in EE-cholestasis. If mrp2 inhibition is indeed a major event in the development of EE-cholestasis, the most important feature of this cholestasis, i.e., a reduction of the BSIF, should not occur in GY/TR$^-$ rats. In this study, we related EE-induced changes in the liver to *in vivo* bile formation, allowing us to interpret the relevance of changes in transporter levels, membrane fluidity and enzyme activities for the inhibitory effect of EE on bile flow.

The recovery of radioactivity in bile after s.c. administration of radiolabeled EE is severely impaired in GY/TR$^-$ rats. This indicates that the EE-metabolites, reported to be mainly glucuronides of 2-hydroxy-EE and 2-methoxy-EE in male rats (39), are normally transported into bile mainly via mrp2. Impaired disposition into bile was partly compensated for by enhanced urinary secretion in GY/TR$^-$ rats, as also described for leukotriene C4 (40), but unlike bile salt sulfates (17) and glucuronides (41). Despite the altered pharmacokinetics of EE in the mutant rats, its effects on plasma lipid levels, hepatic lipid content, hepatic enzyme activities and hepatic plasma membrane composition were similar in both strains. Liver weight was increased by 20% in NW rats after EE-treatment, a phenomenon previously associated with an accelerated hepatic cell proliferation in response to EE (42). This effect was not observed in GY/TR$^-$ rats, in which liver weight under control conditions already is increased in comparison to NW rats.

In NW rats we found changes neither in plasma bile salt and bilirubin concentrations nor in activities of ALAT and ASAT during EE-treatment for up to 5 days. Elevated bile salt levels in rats during EE treatment have been reported in some (13,43) but not in other (44) studies. In our hands, plasma bile salt levels start to increase after 8 days of continuous treatment with 5 mg/kg EE daily (data not shown). Biliary bile salt secretion was unaffected by EE-treatment in both strains, indicating that hepatobiliary transport of endogenous bile salts is not disturbed. Reported effects on 'basal' bile salt secretion in response to EE are varied (1,45-50). However, the S_{RM} of i.v. administered taurocholate is consistently reduced (46,51,52). This is probably due to inhibition of the ATP-dependent transport system responsible for bile salt secretion across the canalicular membrane, as demonstrated by Bossard *et al.* (13). Since the remaining capacity of this system apparently is sufficient to transport endogenous bile salts, this inhibition does not contribute to the reduced bile flow caused by EE in the current study. As plasma bile salt levels and biliary bile salt

secretion were not affected by EE, our results question the relevance of the strongly reduced levels of ntcp found in this and in other studies (37) for the development of EE-cholestasis. Even under conditions of strongly reduced ntcp levels, hepatic uptake is still sufficient to clear bile salts effectively from the portal blood.

To check the possibility that membrane composition and/or fluidity are differentially affected by EE in NW and GY/TR$^-$ rats, we measured these parameters in plasma membranes isolated from livers of our experimental groups. We found that the P_{DPH} in plasma membranes from EE-treated animals was increased. This indicates reduced membrane fluidity, but the effect was only significant in NW rats. As BSIF was inhibited equally in NW and GY/TR$^-$ rats, this finding confirms that decreased membrane fluidity alone is unlikely an important factor in the reduction of BSIF induced by EE. In contrast to the situation reported after 5 days of estrogen administration (5-7), we found no effect on Na^+/K^+-ATPase activity after 3 days despite the decreased membrane fluidity and reduced bile flow. This supports the idea that impaired Na^+/K^+-ATPase activity does not directly contribute to EE-induced cholestasis.

NW and GY/TR$^-$ rats showed a very similar decrease in bile flow after EE administration, exclusively due to a decrease of BSIF. This indicates that the reduction of canalicular mrp2 levels and the reduced GSH secretion found in NW rats can not be the only cause of EE-induced cholestasis. If this were the case, GY/TR$^-$ rats would have been resistant to EE-cholestasis. As bile formation is an osmotic process, there should be reduced net secretion of certain biliary components that account for the decrease in BSIF in both strains. We showed that the secretion of amino acids is not affected by EE-treatment. Increased glycine reabsorption from the bile has been implicated as a factor contributing to EE-cholestasis (13). We found glycine output to be reduced in GY/TR$^-$ rats when compared to NW rats, probably caused by the absence of GSH in their bile. However, glycine output was not affected by EE in NW or GY/TR$^-$ rats, indicating that the reported increase in glycine transport in cLPM isolated from livers of EE-treated rats has no impact on bile formation. Additionally, HCO_3^- concentrations in bile were not different between the EE-treated and untreated rats and HCO_3^- output therefore strongly correlated with bile flow rates, as recently also reported by Alvaro *et al.* in normal rats only (48). So far, we have not been able to identify (a) biliary component(s) that are specifically affected by EE both in NW and GY/TR$^-$ and, therefore, can be considered responsible for the reduced bile flow.

EE-treatment of NW rats resulted in increased secretion of bilirubin into bile, without affecting bilirubin plasma levels. This suggests increased bilirubin production during EE-treatment that must be related to an increased degradation of heme from heme-containing proteins. The nature of these proteins is currently unclear. In the GY/TR$^-$ rats, biliary secretion of bilirubin was not increased during EE-treatment, but plasma levels increased drastically. This indicates that under control conditions bilirubin transport operates at maximal capacity in GY/TR$^-$, leading to disposition into the blood

compartment as a consequence of increased bilirubin production during EE-cholestasis. Nishida *et al.* (53) reported that ATP-dependent transport of conjugated bilirubin is absent in cLPM vesicles from GY/TR rats, while electrogenic transport is unaffected. It may be that secretion measured in the current experiments reflects the maximal capacity of the electrogenic system, but other members of the mrp-family may also be involved.

To further evaluate interference of EE with secretion of organic anions into bile we determined the S_{RM} of the organic anion DBSP in our models. As expected, the S_{RM} for DBSP was decreased upon EE-treatment in NW rats, probably related to reduced levels of mrp2 in the canalicular membrane. In GY/TR rats, in which secretion of DBSP was already decreased under control conditions due to the lack of mrp2, the S_{RM} decreased further upon EE-treatment. This suggests that hepatobiliary transport of organic anions via pathways other than mrp2 is also affected by EE-treatment. Recent data suggest that other members of the mrp family are upregulated in untreated mrp2-deficient rats (54): it may be that these transporters are affected in EE-cholestasis. Alternatively, the high load of organic anions that must be accommodated by these transporters (EE-metabolites, bilirubin, DBSP) in the absence of mrp2 may lead to competition phenomena, resulting in impaired net DBSP transport.

Surprisingly, there was a clear correlation between bile flow and the S_{RM} of DBSP when data of both strains with and without EE were combined. Such a correlation between bile flow and DBSP secretion was also found by Vonk *et al.*(55) during bile salt-induced choleresis. This suggests that not only secretion of organic anions into bile stimulates bile flow, but *vice versa*, bile flow may be a determinant of S_{RM} independent of transporter expression. Decreased bile flow may create a steeper concentration gradient from cell to biliary compartment that affects S_{RM} of DBSP by facilitated reabsorption from bile into liver cells. Such a mechanism may operate also for endogenous compounds that are secreted at or near their S_{RM}. In this respect it is important to note again that S_{RM} for taurocholate is also reduced in EE treated animals (46).

Recapitulating, our data concerning the effects of a relatively short period of EE treatment on bile formation, biliary GSH secretion and canalicular mrp2 levels in normal rats are essentially confirming those of other investigators. However, the observation that the *in vivo* effects of EE on bile formation are similar in normal and mrp2-deficient rats that do not secrete GSH into bile allows the conclusion that inhibition of mrp2 expression and/or activity does not represent 'the' key event in the etiology of EE-induced cholestasis. In 1987, Mary Vore published a comprehensive review on estrogen cholestasis (8) which she ended with the optimistic view that 'the technique of molecular biology should soon provide new insights into the mechanisms of, and appropriate treatment modalities for, estrogen-induced cholestasis'. Almost ten years later we have to conclude that, although much has been learned in the meantime, the actual molecular mechanisms of this type of cholestasis still remains to be defined.

REFERENCES

1. Gumucio JJ, Valdivieso VD. Studies on the mechanism of the ethinylestradiol impairment of bile flow and bile salt secretion in the rat. Gastroenterology 1971;61:339-334.
2. Kreek MJ. Female sex steroids and cholestasis. Sem Liv Dis 1987;7:8-13.
3. Forker EL. The effect of estrogen on bile formation in the rat. J Clin Invest 1969;48:654-663.
4. Iqbal S, Mills CO, Elias E. Biliary permeability during ethinyl estradiol-induced cholestasis studied by segmented retrograde intrabiliary injections in rats. J Hepatol 1985;1:211-219.
5. Davis RA, Kern F, Showalter R, Sutherland E, Sinenski M, Simon FR. Alterations of hepatic Na^+/K^+ ATPase and bile flow by estrogen: effects on liver surface membrane lipid structure and function. Proc Natl Acad Sci 1978;75:4130-4134.
6. Berr F, Simon FR, Reichen J. Ethinylestradiol impairs bile salt uptake and Na^+-K^+ pump function of rat hepatocytes. Am J Physiol 1984;274:G437-G443.
7. Rosario J, Sutherland E, Zaccaro L, Simon FR. Ethinylestradiol administration selectively alters sinusoidal membrane lipid fluidity and protein composition. Biochemistry 1988;27:3939-3946.
8. Vore M. Estrogen cholestasis membranes, metabolites, or receptors? Gastroenterology 1987;93:643-649.
9. Jaeschke H, Trummer E, Krell H. Increase in biliary permeability subsequent to intrahepatic cholestasis by estradiol valerate in rats. Gastroenterology 1987;93:533-538.
10. Boelsterli UA, Rahkit G, Balazs T. Modulation by S-adenosyl methionine of hepatic Na K ATPase, membrane fluidity, and bile flow in rats with ethinyl estradiol-induced cholestasis. Hepatology 1983;3:12-17.
11. Smith DJ, Gordon ER. Role of liver plasma membrane fluidity in the pathogenesis of estrogen-induced cholestasis. J Lab Clin Med 1988;112:697-685.
12. Isaacson Y, van Thiel DH. Increased membrane fluidity and cholestasis: associated but not linked consequences of estrogen treatment. J Lab Clin Med 1988;112:663-666.
13. Bossard R, Stieger B, O'Neil B, Fricker G, Meier PJ. Ethinylestradiol treatment induces multiple canalicular membrane transport alterations in rat liver. J Clin Invest 1993;91:2714-2720.
14. Paulusma C, Bosma PJ, Zaman GJ, Bakker CTM, Otter M, Scheffer GL, Scheper R, et.al. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. Science 1996;271:1126-1127.
15. Oude Elferink RPJ, Meijer DFK, Kuipers F, Jansen PLM, Groen AK, Groothuis GMM. Hepatobiliary transport of organic compounds: multispecific carriers, P-glycoproteins and vesicular pathways. Biochim Biophys Acta 1995;1241:215-268.
16. Trauner M, Arrese M, Soroka CL, Ananthanarayanan M, Koeppel TA, Schlosser SF, Suchy FJ, et.al. The rat canalicular conjugate export pump (mrp2) is down regulated in intrahepatic and obstructive cholestasis. Gastroenterology 1997;113:255-264.
17. Kuipers F, Enserink M, Havinga R, van der Steen A, B, Hardonk MJ, Fevery J, Vonk RJ. Seperate transport systems for biliary secretion of sulfated and unsulfated bile acids in the rat. J Clin Invest 1988;81:1593-1599.
18. Jansen PLM, Peters W, H., Lamers WH. Heriditary chronic conjugated hyperbilirubinemia in mutant rats caused by defective hepatic anion transport. Hepatology 1985;5:573-579.
19. Mikami TT, Nozaki O, Tagaya S, Hosokawa T, Nakura H, Kondou S. The characteristics of a new rat with hyperbilirubinemia syndrome. Congenital Anom 1986;26:250-251.
20. Ito K, Suzuki H, Hirohashi T, Kume K, Shimizu T, Sugiyama Y. Molecular cloning of canalicular multispecific organic anion transporter defective in EHBR. Am J Physiol 1997;272:G16-G22.
21. Oude Elferink RPJ, Ottenhof R, Liefting W, de Haan J, Jansen PLM. Hepatobiliary transport of glutathione and glutathione conjugate in rats with hereditary hyperbilirubinemia. J Clin Invest 1989;84:476-483.
22. Ballatori N, Truong A, T. Relation between the biliary glutathion excretion and bile acid-independent bile flow. Am J Physiol 1989;256:G22-G30.
23. Bouchard G, Yousef IM, Tuchweber B. Decreased biliary glutathione content is responsible for the decline in bile salt-independent flow induced by ethinyl estradiol in rats. Tox Let 1994;74:221-233.
24. Roelofsen H, Vos TA, Schippers IJ, Kuipers F, Koning H, Moshage H, Jansen PLM, Müller M. Increased levels of the multidrug resistance protein in lateral membranes of proliferating hepatocyte-derived cells. Gastroenterology 1997;272:16-22.
25. Kuipers F, Havinga R, Bosschieter H, Toorop GP, Hindriks FR, Vonk RJ. Enterohepatic circulation in the rat. Gastroenterology 1985;88:403-411.
26. Wolters H, Spiering M, Gerding A, Slooff MJH, Kuipers F, Hardonk M, Vonk RJ. Isolation and characterization of canalicular and basolateral plasma membrane fractions from human liver. Biochim Biophys Acta 1991;1069:61-69.
27. Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-275.

28. Goldberg JA, Rutenberg AM. The colorimetric determination of leucine aminopeptidase in urine and serum of normal subjects and patients with cancer and other diseases. Cancer Research 1958;11:283-291.
29. Scharschmidt BF, Keeffe EB, Blankenship NM, Ockner RK. Validation of a recording spectrophpotometric method for measurement of membrane-associated Mg- and NaK-ATPase activity. J Lab Clin Med 1979;93:790-799.
30. Keeffe EB, Scharschmidt BF, Blankenship NM. Studies on the relationships among bile flow, liver plasma membrane NaK-ATPase, and membrane microviscosity in the rat. J Clin Invest 1979;64:1590-1598.
31. Sottocasa GL, Kuylenstierna B, Ernster L, Bergstrand A. An electron-transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. J Cell Biol 1967;32:415-438.
32. De Duve C, Pressman C, Gianetto R, Wattiaux R, Appelmans F. Tissue fractionation studies. 6. intracellular distribution patterns of enzymes in rat-liver tissues. Biochem J 1955;60:604-617.
33. Wolters H, Konings AWT. Radiosensitivity of normal polyunsaturated fatty acid supplemented fibroblasts after depletion of glutathione. Int J Radiat Biol 1984;46:161-168.
34. Murphy GM, Billing BH, Baron DN. A fluorimetric and enzymatic method for the estimation of serum total bile acids. J Clin Pathol 1970;23:594-598.
35. Erikson SK, Jaeckle S, Lear SR, Brady SM, Havel RJ. Regulation of hepatic cholesterol and lipoprotein metabolism in ethinyl estradiol-treated rats. J Lipid Res 1989;30:1763-1771.
36. Koopen NR, Wolters H, Müller M, Schippers IJ, Havinga R, Roelofsen H, Vonk RJ, et.al. Hepatic bile salt flux does not modulate level and activity of the sinusoidal Na^+-taurocholate cotransporter (ntcp) in rats. J Hepatol 1997;(In Press)
37. Simon FR, Fortune J, Iwahashi M, Gartung C, Wolkoff A, Sutherland E. Ethinyl estradiol cholestasis involves alterations in expression of liver sinusoidal transporters. Am J Physiol 1996;271:G1043-G1052.
38. Nishida T, Gatmaitan Z, Che M, Arias IM. Rat liver canalicular membrane vesicles contain an ATP dependent bile acid transport system. Proc Natl Acad Sci 1991;88:6590-6594.
39. James L, Maggs JL, Grabowski PS, Park K. Sex-linked differences in the metabolism and irreversible binding of 17a-ethinylestradiol in the rat. Biochem Pharmac 1983;32:2793-2800.
40. Huber M, Guhlmann A, Jansen PLM, Keppler D. Hereditary defect of hepatobiliary cysteinyl leukotriene elimination in mutants with defective hepatic anion excretion. Hepatology 1987;7:224-228.
41. Kuipers F, Radominska A, Zimniak P, Little JM, Havinga R, Vonk RJ, Lester R. Defective secretion of bile acid-3-O-glucuronides in rats with heridatary conjugated hyperbilirubinemia. J Lipid Res 1989;30:1835-1843.
42. Hornstein B, Stammler L, Bianchi L, Landmann L. Ethinylestradiol increases volume and decreases sinusoidal membrane surface in the rat liver: A stereological analysis. Hepatology 1992;16:217-223.
43. Azer SA, Canfield PJ, Stacey NH. Hepatoprotection in ethinylestradiol-treared rats is provided by tauroursodeoxycholic acid, but not by ursodeoxycholic acid. J Gastr Hepat 1995;10:261-269.
44. Hillstrom J, Duane WC, Eckfeldt JH, Furne J, Levitt MD. Lack of benefit of ursodeoxycholic acid in drug-induced cholestasis in the rat. Proc Soc Exp Biol Med 1992;200:122-126.
45. Accatino L, Pizarro M, Solis N, Koenig CS, Vollrath V, Chianale J. Modulation of hepatic content and biliary excretion of P-glycoproteins in hepatocellular and obstructive cholestasis in the rat. J Hepatol 1996;25:349-361.
46. Gumucio JJ, Accatino L, Macho AM, Contreras A. Effect of phenobarbital on the ethynyl estradiol-induced cholestasis in the rat. Gastroenterology 1973;65(4):651-657.
47. Tuchweber B, Perea A, Carrillo MC, Villaqlon L, Lemáy J, Yousef IM. Low protein diet protects against ethinyl estradiol-induced cholestasis. Nutrition Research 1994;14:707-716.
48. Alvaro D, Gigliozzi A, Piat C, Carli L, Fraioli F, Romeo R, Francia C, et.al. Inhibition of billiary bicarbonate secretion in ethinyl estradiol-induced cholestasis is not associated with impaired activity of the Cl^-/HCO_3^- exchanger in the rat. J Hepatol 1997;26:146-157.
49. Rodriguez J.V., Torres AM, Lunazzi G, Tiribelli C. Effect of ethinylestradiol and epomediol on bile flow and biliary lipid composition in rat. Biochem Pharmacol 1992;43:1289-1293.
50. Goldsmith MA, Jones AL, Underdown BJ, Schiff MJ. Alterations in protein transport events in rat liver after estrogen treatment. Am J Physiol 1987;253:G195-G200.
51. Kern F, Eriksson H, Curstedt T, Sjoval J. Effect of ethynyl estradiol on biliary excretion of bile acids, phosphatidylcholines, and cholesterol in the bile fistula rat. J Lipid Res 1977;18:623-634.
52. Arrese M, Pizarro M, Solis N, Koenig C, Accatino L. Enhanced biliary excretion of canalicular membrane enzymes in ethynylestradiol-induced cholestasis. Effects of ursodeoxycholic acid administration. Biochem Pharmac 1995;50:1223-1232.
53. Nishida T, Gatmaltan Z, Roy-Chowdhry J, Arias IM. Two distinct mechanisms for bilirubin glucuronide transport by rat canalicular membrane vesicles. J Clin Invest 1992;90:2130-2135.
54. Hirohashi T, Niinuma K, Ito K, Suzuki H, Kume K, Shimizu T, Sugiyama Y. Characterization of ATP dependent organic anion transport system on rat canalicular membrane maintained in EHBR. Hepatology 1996;24:305(Abstract)

55. Vonk RJ, Danhof M, Coenraads T, van Doorn AB, Keulemans K, Scaf AH, Meijer DFK. Influence of bile salts on hepatic transport of dibromosulphthalein. Am J Physiol 1979;273:E524-E534.

Chapter 3

Differential effects of 17α-ethinylestradiol on the neutral and acidic pathways of bile salt synthesis in the rat.

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ABSTRACT

Administration of 17α-ethinylestradiol (EE) affects BS metabolism in rats. The aim of this study was to evaluate the effects of EE on the neutral and acidic biosynthetic pathways of BS synthesis. Effects of EE (5 mg/kg, 3 days) on bile salt pool composition and on synthesis of individual BS *in vivo* were studied by gas chromatography-mass spectrometry, in rats with an intact enterohepatic circulation and in rats with long-term bile diversion to induce BS synthesis. Outcomes were related to hepatic activities and expression levels of cholesterol 7α-hydroxylase (CYP7A) and sterol 27-hydroxylase (CYP27), as well as of other enzymes involved in BS synthesis, i.e. sterol 12α-hydroxylase and lithocholate 6β-hydroxylase. BS pool size was decreased by 27% but total BS synthesis was not affected by EE in intact rats. The contribution of cholate to the pool was decreased by 20%. Synthesis of cholate was reduced by 68% in EE-treated rats, while that of chenodeoxycholate was increased by 60%. The recently identified Δ^{22}-isomer of β-muricholate contributed for 5.4% and 18.3 % ($p<0.01$) to the pool in control and EE-treated rats, respectively. This BS could not be detected in bile after exhaustion of the pool, indicating that Δ^{22} β-muricholate is not a primary BS. In contrast to the situation in intact rats, a clear reduction of BS synthesis was found in bile-diverted EE-treated rats. Yet, biliary BS composition was only minimally affected. Activity of CYP7A was decreased by EE in both intact and bile-diverted rats, whereas the activity of the CYP 27 was not affected. Hepatic mRNA levels of CYP7A were only significantly reduced by EE in bile-diverted rats; CYP27 mRNA levels were not affected by EE. In addition, mRNA levels of sterol 12α-hydroxylase, lithocholate 6β-hydroxylase as well as of HMG-CoA synthase were increased after bile diversion and suppressed by EE. This study shows that EE-induced intrahepatic cholestasis in rats is accompanied by changes in BS pool size and composition, resulting from a selective inhibition of the neutral pathway of BS synthesis. In intact rats BS synthesis via the alternative pathway takes over when the neutral pathway is suppressed: simultaneous impairment of other enzymes in these biosynthetic pathways and of cholesterol synthesis may contribute to overall effects of EE on BS synthesis.

INTRODUCTION

The synthetic estrogen 17α-ethinylestradiol (EE) induces cholestasis in rodents, mainly by reducing the bile salt-independent fraction of bile flow (BSIF) (1,2). The mechanisms underlying the decrease in bile flow are not yet clear (3). In addition to reduced BSIF, several authors have reported that EE may also affect bile salt-dependent bile formation (BSDF) (2,4,5). This effect has been attributed to reduced hepatic BS synthesis (4,6) and to impaired activities of hepatic transport systems involved in vectorial transport from blood to bile (7-9). With respect to the first, reduced biliary BS secretion and altered biliary BS composition in EE-treated animals have been reported (4,5,10). In particular, the contribution of chenodeoxycholate and β-muricholate to the BS pool appears to increase at the expense of cholate. In a previous study (10), we found that conversion of endocytozed lipoprotein cholesterol to cholate was completely abolished in EE-treated rats. The metabolic basis for the changes in BS synthesis induced by EE are largely unknown. Earlier studies have shown that EE inhibits the activity of the cholesterol 7α-hydroxylase (11,12), the enzyme catalyzing the first step of the so-called neutral pathway of BS biosynthesis. Since then, however, it has become clear that an acidic pathway, initiated by 27-hydroxylation of cholesterol by the mitochondrial sterol 27-hydroxylase, represents a quantitative important route for BS synthesis (13-15). Recently, data have been reported to indicate that in situations where the "classical" neutral pathway is specifically suppressed, the acidic pathway becomes more important for maintenance of hepatic BS synthesis (16,17). It is not known whether EE differentially affects both pathways. In addition, peroxisomal formation of a Δ^{22} isomer of muricholate has been suggested as a novel further downstream pathway in BS synthesis in rats (18,19). Whether and to what extent formation of this species is affected under cholestatic conditions is not known. Finally, EE has strong impact on hepatic cholesterol synthesis (11,20-22). As the contribution of newly synthesized cholesterol to formation of individual BS may vary under different conditions (23-25), altered cholesterol synthesis may also affect BS synthesis.

To assess the quantitative contribution of the major pathways to hepatic BS synthesis in EE-treated rats we related *in vivo* BS synthesis to the specific activities and expression levels of cholesterol 7α-hydroxylase (CYP7A) and sterol 27-hydroxylase (CYP27). In addition, mRNA levels of sterol 12α-hydroxylase and lithocholate 6β-hydroxylase, key enzymes in the formation of cholate and β-muricholate, respectively, were determined, as well as those of HMG-CoA synthase as a key enzyme in cholesterol synthesis. Experiments were performed in rats with an intact enterohepatic circulation and in rats with prolonged bile diversion. Bile diversion leads to pool depletion and to upregulation of hepatic BS synthesis, thereby enabling us to directly assess the effects of EE on synthesis of the individual BS species and to relate these effects to hepatic enzyme activities.

MATERIALS AND METHODS

Materials. 17α-Ethinylestradiol (EE) was purchased from Sigma Chemicals (St. Louis, MO, USA). NADPH, isocitrate-dehydrogenase was obtained from Boehringer Mannheim (Mannheim, Germany). Cholesterol oxidase was obtained fron Calbiochem (La Jolla,CA, USA). All other chemicals were of reagent grade or the highest purity commercially available.

Animals. Male Wistar rats (Harlan Laboratories, Zeist, The Netherlands) weighing 290-330 g were used for these studies. Animals were kept in a light- and temperature-controlled environment and had free access to lab chow and tapwater throughout the experiments. The animals received humane care and experimental protocols complied with the local guidelines for use of experimental animals.

To study the effects of EE on bile formation and composition under conditions with an intact enterohepatic circulation, rats were equipped with permanent catheters in bile duct and duodenum as described in detail elsewhere (26). Both catheters were immediately connected to each other to maintain an intact enterohepatic circulation. Subcutaneous EE (5 mg/kg) or solvent (1,2-propanediol) injections were given for 3 days, starting four days after surgery, i.e., after animals had regained their preoperative body weights. After three days of treatment, the connection between both catheters was interrupted and bile was collected for 6 hours in 30 min intervals by means of a fraction collector. Bile volume was determined gravimetrically and samples were immediately stored at -20 °C for later analysis. Separate groups of rats were used for the isolation of hepatic microsomes, mitochondria, total RNA and for collection of blood.

In order to study the effects of EE on bile formation and composition after long-term bile diversion, when BS synthesis is maximally upregulated, rats where equipped with a permanent catheter in the bile duct only. Bile was diverted for 5 days, prior to administration of EE or the solvent for three days. These animals were allowed to drink 0.9% NaCl to compensate for loss of electrolytes via bile. At day 8, bile samples were continuously collected by means of a fraction collector for 24 hr in 90 min intervals. After the bile sampling, the animals were anesthetized with halothane. Blood was sampled by means of a cardiac puncture and the liver was removed for isolation of total RNA, microsomes and mitochondria.

Analyses. BS in plasma and bile were determined by an enzymatic fluorimetric assay (27). Plasma triglycerides, plasma- and hepatic cholesterol were measured enzymatically using commercially available kits (Boehringer Mannheim, Mannheim, Germany). Aspartate transaminase (AST), alanine transaminase (ALT) and bilirubin in plasma were assessed by standard laboratory techniques.
Bile salt composition was studied by gas chromatography and gas chromatography / mass spectrometric techniques as described earlier for human bile (28). Briefly 5 - 50 μl bile was subjected

to enzymatic hydrolysis with cholylglycine hydrolase. The free bile acids formed were extracted with C18 solid phase extraction, methylated and silylated. The methyl-TMS derivatives were separated on a 25m x 0.25 mm OV-1701 column (CP Sil19 CB, Chrompack Int., Middelburg, The Netherlands). As a modification coprostanol was used as internal standard for the purpose of quantitation applying GC only. Identification of bile acids was performed by GC/MS (SSQ7000, Finnigan MAT, San Jose, CA, U.S.A.) using the same GC separation system. Full scan data were recorded from m/z 50 - 850 and mass spectra were compared with reference spectra for definitive identification. In the absence of reference spectra, a tentative identification was done based on spectral information (19).

Preparation of microsomes and mitochondria. For the isolation of microsomes and mitochondria, livers were perfused with cold saline, removed and 5 gram of liver tissue was stored in 250 mM sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4. All procedures were carried out at 4°C. Livers were cut into small pieces with scissors and homogenized in the same buffer using a potter. The homogenate was centrifuged for 10 min at 800 g, and the supernatant was then centrifuged 12 min at 8500 g. The thus obtained supernatant was used for isolation of microsomes and the pellet for the isolation of mitochondria.
For the isolation of microsomes the supernatant was centrifuged for 70 min at 100000 g. The pellet was resuspended by means of a potter in 100 mM sucrose, 100 mM potassium phosphate, 2 mM EDTA and 5 mM DTT, pH 7.4 and centrifuged for 1 h at 100000 g. Microsomes were resuspended in the same buffer and frozen quickly in fluid N_2 in small aliquots and stored at -80°C.

The mitochondrion-enriched pellet was resuspended by means of a potter in 250 mM sucrose, 10 mM Tris , pH 7.4 and centrifuged for 12 min at 8500 g. This procedure was repeated 3 times. The final mitochondrial pellet was resuspended in this buffer and stored at -80°C. Protein concentration was measured according to Lowry (29)

Assay of cholesterol 7α-hydroxylase and sterol 27-hydroxylase enzyme activity. Enzyme activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase in isolated liver microsomes and mitochondria were determined essentially according to Chiang (30) measuring conversion of cholesterol into 7α- and 27-hydroxycholesterol, respectively. In short, 750 μg of protein of either microsomal or mitochondrial protein was incubated in 1 ml of buffer containing 0.1 M potassium phosphate pH 7.2, 50 mM NaF, 5 mM DTT, 1 mM EDTA, 20% glycerol (w/v) and 0.015 % (w/w) CHAPS. Twenty μl of 1 mg cholesterol in 45% (w/v) hydroxypropyl-β-cyclodextrin was added and the mixture was incubated with agitation for 10 min at 37 °C. Then 200 μl of a regenerating system was added containing 10 mM sodium isocitrate, 10 mM $MgCl_2$, 1 mM NADPH and 0.15 U isocitrate-dehydrogenase at 37 °C. After 20 min of incubation 60 μl of a stop solution containing 20% sodium cholate and 1μg 20α-hydroxycholesterol, which served as an internal standard, was added. Steroid products were oxidized for 45 min with 100 μl buffer containing: 0.1 % cholesterol oxidase, 10mM

ethanol. Cholesterol metabolites from this reaction mixture were extracted in petroleumether and the ether layer was evaporated under a stream of nitrogen. Residues resuspended in a mixture of 60% acetonitril, 30% methanol and 10% chloroform (v/v) were analyzed by using C-18 reverse phase HPLC on a Tosohaas TSK gel-ODS 80TM column equilibrated with 70% acetonitril and 30% methanol at a flow rate of 0.8 ml/min. The amount of products formed was determined by monitoring the absorbance at 240 nm. Peaks were integrated using Data Control software (Cecil Instruments, UK).

Determination of mRNA levels. Total RNA was isolated according to Chomczynski (31). Determination of steady state mRNA levels for CYP7A, CYP27, 12α-hydroxylase, lithocholic acid 6β-hydroxylase, HMG-CoA synthase and LDL receptor by Northern blot and dot blot and hybridization conditions were performed as described previously (32-34). 18S ribosomal RNA was used as an internal standard to correct for differences in amounts of total RNA applied to the gel. mRNA levels were quantified by phospho-imager analysis (Fuij Fujix Bas 1000) by using the program TINA version 2.08c.

Calculations and statistics. Output rates of BS were determined by multiplying bile flow with BS concentrations, after correction for the dead space of the tubing system. Values are expressed as mean ± SD. Significance of difference between two groups was assessed by means of Mann-Whitney nonparametric test at $p<0.05$ level of significance.

RESULTS

Animal characteristics. EE treatment had no significant effects on body weight in intact or bile-diverted rats (Table 1). The liver-to-body weight ratio increased significantly upon EE treatment in both conditions. Table 2 shows the effects of EE treatment for three days on plasma markers of liver function. Aspartate transaminase, alanine transaminase and bilirubin levels in plasma were not significantly changed. The plasma BS concentration increased significantly in intact rats upon EE treatment, i.e. from 36 ± 12 to 77 ± 22 μM. As expected, BS levels were at the lower limits of detection in the untreated bile-diverted rats and did not increase after EE administration. Treatment with EE led to significant reductions in plasma cholesterol and triglyceride levels in both experimental models.

	Body weight (g)	*Liver weight (g)*	*Liver (% Body weight)*
Intact, control	325.0 ± 49.4	14.2 ± 2.6	4.3 ± 0.2
Intact, EE	332.6 ± 39.0	17.5 ± 2.5	5.3 ± 0.2*
Bile diverted, control	322.0 ± 23.9	13.4 ± 0.9	4.2±0.3
Bile diverted, EE	298.3 ± 24.3	14.3 ± 0.7	4.8 ± 0.4*

Intact and bile diverted rats were treated with EE (5mg/kg) for three consecutive days. At 24 h after the last injection, animals were weighed, anesthesized with halothane and a large blood sample was collected by cardiac puncture. Subsequently the liver was removed and weighed.
Data are given as means ± SD, n=3-5 per group
** significantly different ($p < 0.05$) from respective control*

Table 1 Body- and liver weights in control and EE-treated intact and bile diverted rats.

	AST (IU/L)	*ALT (IU/L)*	*Bilirubin (μM)*	*Bile Salt (μM)*	*Cholesterol (mM)*	*Tiglyceride (mM)*
Intact, control	89 ± 16	41 ± 2	5.5 ± 1.7	36 ± 12	1.33 ± 0.66	1.34 ± 0.66
Intact, EE	115 ± 50	40 ± 11	5.2 ± 1.1	77 ± 22 *	0.32 ± 0.05 *	0.21 ± 0.11 *
Bile diverted, control	222 ± 81	52 ± 14	8.3 ± 5.9	4 ± 0	1.39 ± 0.35	1.42 ± 0.65
Bile diverted, EE	112 ± 74	30 ± 6	4.8 ± 0.5	2 ± 1	0.39 ± 0.14 *	0.33 ± 0.27 *

See legend table 1 for experimental details. Data are given as means ± SD, n=3-5 per group
** significantly different $p < 0.05$ from respective control*

Table 2. Effect of EE treatment for 3 days on plasma markers in intact and bile diverted rats.

Bile formation. Figure 1A shows bile flow during 6 hours following interruption of the enterohepatic circulation of intact rats (left panel) and during a 24 hr period after 8 days of bile diversion (right panel). EE treatment significantly decreased bile flow in intact rats, which, as shown previously (3), is mainly caused by reduction of the BSIF. EE also decreased bile flow in rats with long-term bile diversion.

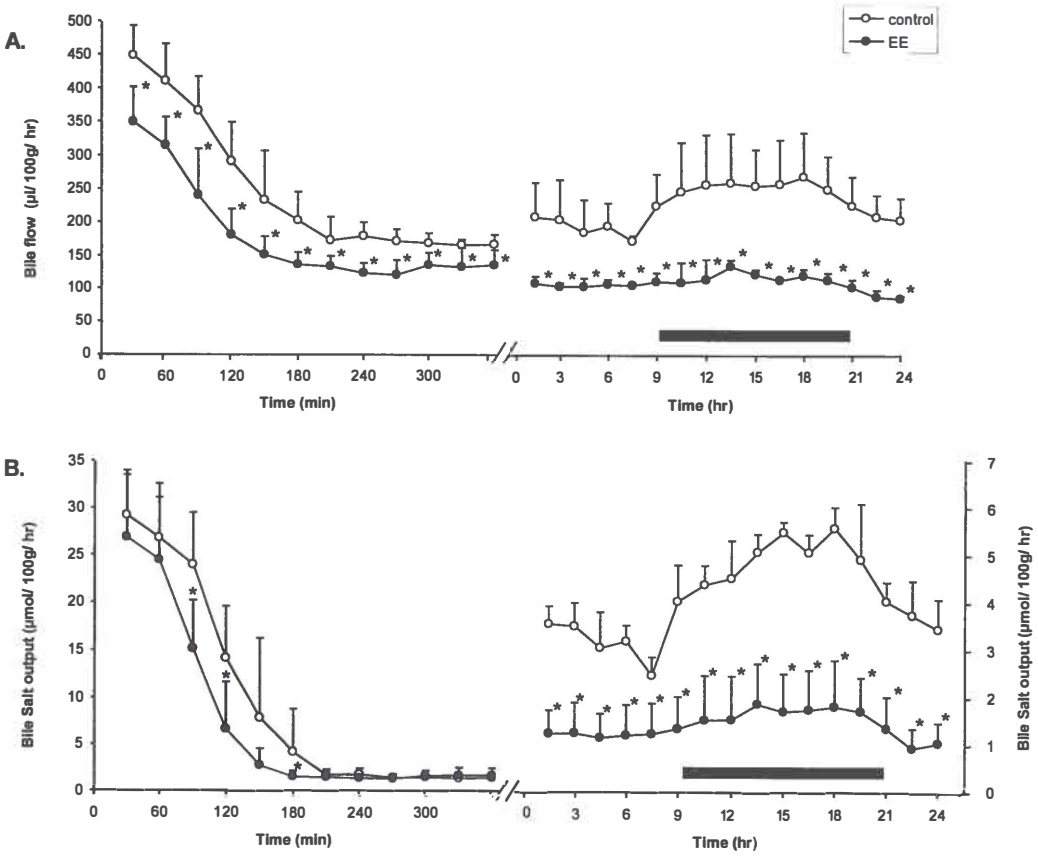

Fig 1. The effect of EE on bile flow (A) and BS output (B) in intact rats during 6 h following interruption of the enterohepatic circulation (left panel) and the effect of EE during 24 h after 8 days of bile diversion (right panel). *In intact rats bile was collected in 30 min fractions for 6 hours after interruption of the enterohepatic circulation. In 8 day bile-diverted rats bile was collected continuously during 24 hours in 90 min fractions.*

Horizontal bars indicate the dark period. Values are means ±SD of 3-6 rats per group.

** significantly different from respective control ($p<0.05$*

In this condition, the well-established diurnal variation in bile formation was absent. BS output was decreased upon EE treatment in both intact and bile-diverted rats (Figure 1B). BS poolsizes, as calculated from the wash-out curves in the intact rats were 53.0 ± 17.2 µmol/100g in the controls and 37.0 ± 12.9 µmol/100g in the EE-treated rats respectively ($p< 0.05$). The BS synthesis rate, determined at the nadir of the wash-out curve, was not affected by EE treatment, i.e. 1.54 ± 0.16 µmol/h/100g and 1.57 ± 0.16 µmol/h/100g in control and EE-treated rats, respectively. After 8 days of bile diversion, BS synthesis rate increased to 3.37 ± 0.52 µmol/h/100g in control rats at day time, whereas in the EE-treated bile-diverted animals synthesis was significantly lower, 1.23 ± 0.04 µmol/h/100g. This figure

also shows that the characteristic increase in BS output during the dark period in bile-diverted control rats (35) was absent in the EE-treated rats. These results imply that, after long-term bile diversion, the estrogen also impairs the magnitude of the BSDF.

BS species	*BS Pool*		*BS Synthesis*	
	control (%)	*EE (%)*	*control (%)*	*EE (%)*
LC	1.1 ± 0.2	1.5 ± 0.6	2.8 ± 1.1	1.8 ± 1.3
DC	3.5 ± 1.3	5.2 ± 3.2	11.0 ± 3.1	15.5 ± 7.4
C	67.0 ± 3.7	50.8 ± 12.2 *	36.9 ± 4.6	11.8 ± 1.7 **
CDC	9.6 ± 3.5	8.3 ± 4.4	20.9 ± 4.5	33.4 ± 8.3 **
HDC	5.9 ± 1.9	8.8 ± 3.8	14.0 ± 3.7	18.1 ± 2.8
UDC	2.2 ± 1.0	0.2 ± 0.5 **	0.9 ± 0.4	1.6 ± 0.9
β-MC	5.5 ± 2.8	7.0 ± 1.9	12.1 ± 2.4	16.1 ± 4.8
ΔβΔ22MC	5.4 ± 1.7	18.3 ± 3.8 **	1.2 ± 0.5	1.7 ± 0.7

After interruption of the enterohepatic circulation, 30 min bile samples were collected. Pool composition was determined in the 0-30 sample. Synthesis was measured at 300-330 min after the interruption i.e., after exhaustion of the BS pool. Data are given as means ± SD, n= 4-5 per group

** significantly different (p< 0.05),*

*** (p<0.01) from respective control*

Table 3. The effect of EE-treatment on bile salt pool composition and on composition of newly synthesized bile salts immediately after exhaustion of the circulating pool.

The relative contribution of the individual BS present in the pool was assessed by GC and GC-MS analysis (Table 3). In the BS pool, the contribution of cholate was decreased from 67.0 ± 3.7 to 50.8 ± 12.2 %, probably caused by a decreased cholate synthesis, as determined after pool depletion. On the other hand, synthesis of the other primary BS, i.e. CDC and β-MC were relatively increased by EE. Secondary BS, i.e., DC and LC were present in bile in low concentrations after 6 h pool depletion, probably reflecting their slow entry into the bloodstream from the colon. Their contribution was not affected by EE. The recently identified (18,19) Δ^{22} isomer of β-muricholate comprised 5.4 % of the BS pool in control rats and 18.3 % in EE-treated rats. The identity of this unique rodent BS was confirmed by GC-MS, based upon the unique combination of fragment ions m/z 195, 285 (typical for muricholic acids) and 367, 456 and 546 (Figure 2) indicating a combination of a 3α,6α or 6β,7β- trihydroxy bile acid and a single double bond. According to Setchell *et al.* (19) the double bond is located at the C22 position. A similar Δ22 ω-muricholic acid could be identified based on the similar fragmentation pattern and the retention time shift relative to ω-muricholic acid which is comparable with the retention time shift of Δ22 β-muricholic acid relative to β-muricholic acid.

After exhaustion of the pool, only traces of Δ^{22}-β-muricholate could be detected in bile of both control and EE-treated rats. After long-term bile diversion, the isomer was not detectable at all (Table 4). This implies that this peroxisomal β-oxidation product of β-MC (18) can not be considered as a primary BS in rats.

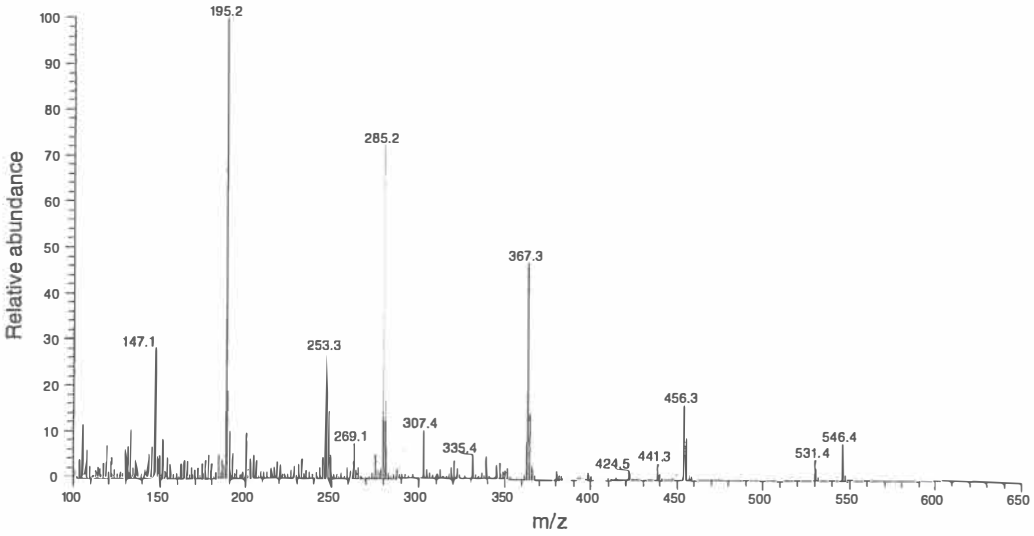

Fig 2. GC-MS spectrum of Δ 22-β-muricholate

BS species	*control (%)*	*EE (%)*
C	51.0 ± 7.2	46.3 ± 6.2
CDC	33.9 ± 3.2	28.4 ± 8.7
UDC	2.8 ± 0.9	4.0 ± 0.2
βMC	13.0 ± 5.9	21.2 ± 4.8

90 min bile samples were taken from long-term bile-diverted rats after 3 days of EE treatment. A sample at the mid-light period i.e. from 12-13.30 AM was used for determining BS composition. Data are given as means ± SD, n=3-5 per group

Table 4. The effect of EE on BS composition in long term bile diverted rats.

Table 4 shows BS composition in 8 day bile-diverted rats when BS synthesis is maximally upregulated (26). As the intestinal BS pool of these rats has been depleted, only primary BS are present in bile. The decrease in the relative cholate synthesis seen in EE-treated intact rats was not found after long-term bile diversion. Also the other BS were not significantly affected by EE, although the relative contribution of β-MC tended to be increased by treatment with EE.

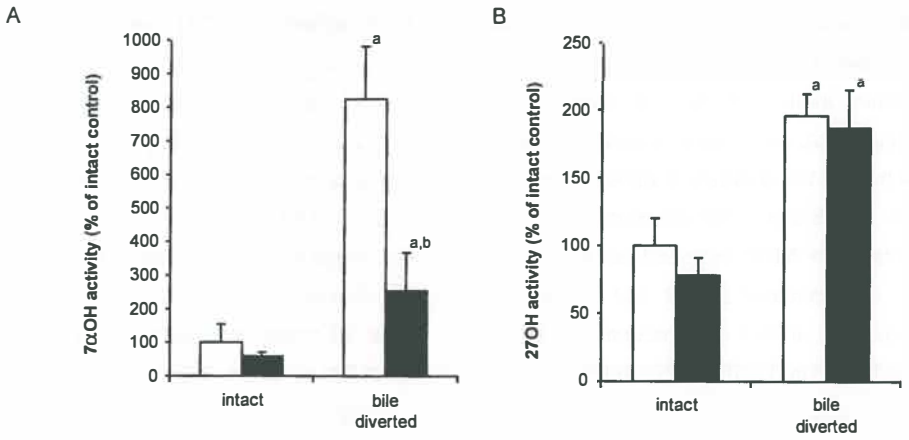

Fig 3. Microsomal cholesterol 7α-hydroxylase (a) and mitochondrial sterol 27-hydroxylase (b) activities in intact and bile-diverted solvent-treated (control) and EE-treated rats .
Microsomes and mitochondria were prepared from livers of the experimental groups harvested at 9 AM and enzyme activities were measured as described in the Materials and Methods section. White bars indicate solvent-treated control groups and black bars indicate EE-treated groups. Values are means ± SD for 3-6 rats per group and expressed as percentage of the untreated, intact control group. The 100% value is 2.78 ± 1.51 nmol/mg/hr for cholesterol 7α-hydroxylase and 1.28 ± 0.26 nmol/mg/hr for sterol 27-hydroxylase.
a = significantly different p<0.05 from intact control
b= significantly different p<0.05 from bile diverted control

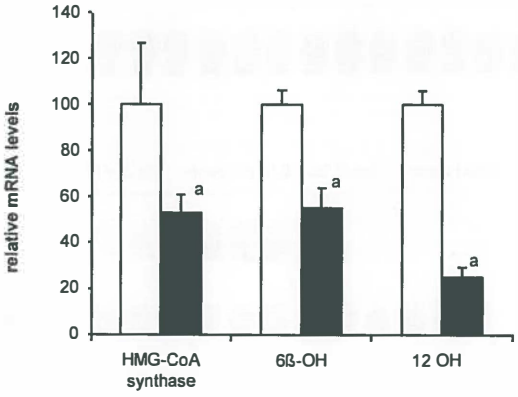

Fig 4. Relative mRNA levels of cholesterol 7α-hydroxylase (CYP7), sterol 27-hydroxylase (CYP27) and LDL receptor (LDLr) in intact and bile-diverted solvent-treated (control) and EE-treated rats *Liver material used for RNA isolation was harvested at 9 AM. White bars indicate solvent treated control groups and black bars indicate EE-treated groups. mRNA levels were quantified relative to 18S RNA signal. Data are mean ± SD for 3-5 rats per group, expressed as percentage of the intact solvent-treated control group*
a = significantly different p<0.05 from intact control.
b= significantly different p<0.05 from bile diverted control.

Activities and mRNA levels of cholesterol 7α-hydroxylase (CYP7A) and sterol 27-hydroxylase (CYP27). In order to gain insight into the metabolic background of the changes in BS composition induced by EE, the activities of the cholesterol 7α-hydroxylase and the sterol-27-hydroxylase were determined in isolated hepatic microsomes and mitochondria, respectively. Figures 3a and b show the activities of these enzymes, expressed as percentage of the solvent-treated intact controls. After 8 days of bile diversion, i.e., without administration of EE, the activity of cholesterol 7α-hydroxylase was 8-fold increased, as shown previously (36). Upon EE treatment the activity of this enzyme was decreased by 44% and 70%, in intact and bile-diverted rats, respectively. It should be noted that the activity of cholesterol 7α-hydroxylase in the EE-treated bile-diverted rats was still increased compared with the control situation. After 8 days of bile diversion, the activity of sterol 27-hydroxylase was increased by 100%. EE treatment did not change activity of this enzyme, in intact or bile-diverted rats. These results therefore indicate that EE treatment affects the neutral but not the acidic pathway in BS synthesis at the level of enzymes catalyzing their initial steps.

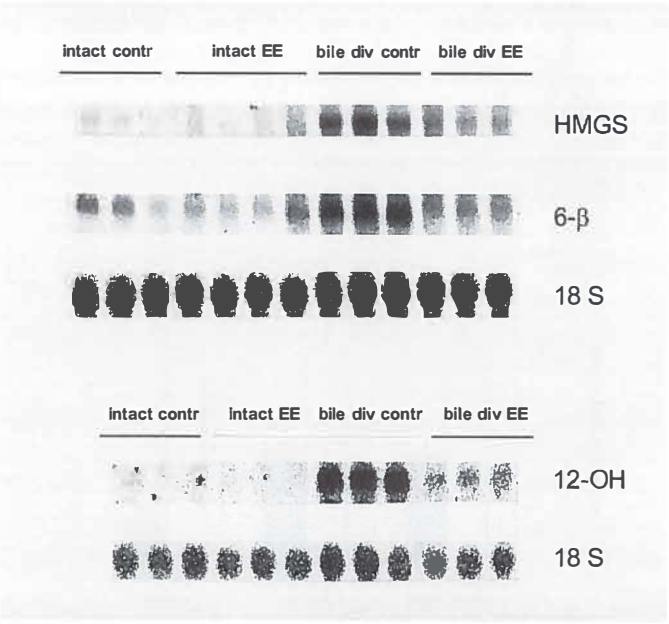

Figure 5. Northern blot analysis of lithocholate 6β-hydroxylase, sterol 12α-hydroxylase and HMG-CoA synthase in intact and bile diverted rats treated with solvent or EE (a) and relative mRNA levels in 8 day bile-diverted rats with and without EE treatment (b). *Liver material used for RNA isolation was harvested at 9 AM. White bars indicate solvent treated control groups and black bars indicate EE-treated groups. mRNA levels were quantified relative to 18S RNA signal. Data are mean ± SD for 3-5 rats per group, expressed as percentage of the intact solvent-treated control group*

a = significantly different $p<0.05$ from intact control

To investigate the level of EE-interaction with BS synthesis, we determined steady state mRNA levels of *CYP7A* and *CYP27*. Figure 4 shows that mRNA levels of *CYP7* and *CYP27* as well as of the LDL receptor, measured as a control signal, increased upon bile diversion. The *CYP7A* mRNA levels were not significantly lowered by EE in intact rats but clearly decreased in bile-diverted rats. *CYP27* mRNA levels, however, were not significantly affected by EE in either situation. The levels of LDL receptor mRNA were increased upon EE treatment as reported previously (20,21,37,38), both in intact and bile-diverted animals.

The mRNA levels of sterol 12α-hydroxylase essential for cholate synthesis and of lithocholate 6β-hydroxylase, involved in β-muricholate formation, were clearly increased in bile diverted rats compared to intact animals and markedly down-regulated by EE-treatment (Figure 5). Furthermore, HMG-CoA synthase mRNA levels were increased after bile diversion, as expected, and clearly reduced by EE treatment, confirming earlier reports (11,21) showing reduced cholesterol synthesis in EE-treated rats.

DISCUSSION

In the present study, we have evaluated the effects of EE on expression and activity of key enzymes of the neutral and acidic pathways of BS synthesis, i.e. cholesterol 7α-hydroxylase and sterol 27-hydroxylase, respectively, as well as on BS pool composition and synthesis *in vivo* in rats. This approach allows us to compare enzyme activities and mRNA levels directly to the "end result" in bile. Rats were treated with the estrogen for 3 days. Based on previous experiments (3), we anticipated that, with this treatment schedule, changes observed would be attributable to EE rather than to secondary effects of full-blown cholestasis. As demonstrated in several studies (4,6,39-41), the presence of cholestasis in itself also affects BS synthesis in rats. Based on the minimal changes in plasma bilirubin, BS and transaminases it can be concluded that, although bile flow was markedly reduced in EE-treated animals, there was no accumulation of bile components in the plasma nor was liver damage induced in this experimental set-up. Previous studies in our laboratory have shown that long-term bile diversion leads to a 5-6 fold increase in hepatic HMG-CoA reductase activity (36) and a 2-fold increase in total body cholesterol synthesis (42). Simultaneously, cholesterol 7α-hydroxylase activity (7-fold) and BS synthesis (2-fold) were found to be increased (36). Results of the present study confirm these results and show that CYP7A mRNA levels are 4-fold increased by long-term bile diversion. The results of the current study show that interruption of the enterohepatic circulation in rats for 8 days also leads to a 2-fold increase in sterol 27-hydroxylase activity and a 2.5 fold increase in CYP27 mRNA levels. These results support previous findings in cholestyramine-fed rats and *in vitro* studies in cultured rat hepatocytes, demonstrating feed-back regulation of sterol 27-hydroxylase activity by BS at a transcriptional level (24,43,44). In contrast to the situation in rats, cholesterol 7α-

hydroxylase and sterol 27-hydroxylase do not appear to be coordinately regulated by recirculating BS in the rabbit liver (17,45), delineating the remarkable inter-species differences in regulation of BS metabolism.

In addition to the anticipated effects on *CYP7A* and *CYP27* as well as on *HMGS* expression it is shown for the first time that mRNA levels of the sterol 12α-hydroxylase and lithocholate 6β-hydroxylase are markedly increased in the bile diverted rat, indicating that BS exert regulatory actions at multiple sites of their biosynthetic pathways. Alternatively, it may be that hepatic accumulation of BS precursors, due to increased activities of the rate-limiting enzymes, increases gene transcription and/or mRNA stability of enzymes catalyzing conversions further down-stream in the biosynthetic cascade. It is also interesting to note that the well-established diurnal variation of BS synthesis in bile depleted rats (26) is completely abrogated by EE. As this diurnal rhythm is thought to be mediated by glucocorticoids (46) it is tempting to speculate that EE renders the BS synthetic cascade insensitive to stimulatory actions of glucocorticoids. The mechanism underlying this resistance remains to be elucidated.

Administration of EE to rats with intact enterohepatic circulation leads to compromised BS pool size, clear changes in pool composition and in the relative amounts of *de novo* synthesized BS, but has no effects on total BS synthesis. In contrast, in bile-diverted animals, there is a marked reduction in hepatic BS synthesis without clear-cut changes in BS composition. In both situations, EE reduced cholesterol 7α-hydroxylase activity and *CYP7A* mRNA levels, but had no effect on sterol 27-hydroxylase activity or *CYP27* mRNA levels. In the bile-diverted rats mRNA levels of HMG-CoA synthase, sterol 12α-hydroxylase and lithocholate 6β-hydroxylase were also clearly suppressed by EE. Taken together, this suggests that EE affects expression of endoplasmic reticulum localized enzymes but is without effect on a mitochondrial system involved in BS metabolism, as investigated in this study. At first sight, these findings seem to indicate that EE rather selectively suppresses the contribution of the neutral pathway of BS synthesis. In the intact rats only, this is apparently compensated for by increased flux via the acidic pathway. In spite of the unchanged BS synthesis rate in intact rats however, BS pool size was significantly reduced. This may indicate that EE-treated rats are unable to upregulate hepatic BS synthesis adequately to compensate for fecal BS loss: it may be that EE treatment down-regulates the recently identified intestinal Na^+-dependent BS transporter (ibst) similar to its reported effects on expression of the hepatic Na^+-dependent BS transporter (ntcp) (8), leading to less effective conservation of BS in the enterohepatic circulation.

Our data also indicate that, in particular in bile-diverted animals, steps prior to or beyond the initial 27-hydroxylation may become rate-limiting in the alternative pathway, leading to a reduced synthesis in a situation when sterol 27-hydroxylase is markedly upregulated. This suggestion is supported by the absence of significant changes in biliary BS composition under these conditions. Another factor that may become rate-limiting in bile diverted animals is the supply of substrate, i.e. of (newly synthesized)

cholesterol. We (25) and others (23) have shown that the contribution of *the novo* synthesized cholesterol to BS amounts up to 12 % in the intact rat with low BS synthesis and up to 40 - 50% in bile diverted rats. This shift in relative contribution of newly synthesized cholesterol to BS synthesis is most likely due to the fact that both synthetic processes are physically separated under normal conditions, i.e., are localized in different hepatocyte populations (24). This zonal distribution is largely lost when BS synthesis and cholesterol synthesis are derepressed by interruption of the enterohepatic circulation or cholestyramine-feeding (24). As EE inhibits hepatic cholesterol synthesis (11,20,21), as confirmed in our study by decreased levels of *HMGS* mRNA in livers of EE-treated rats, it is conceivable that reduced substrate availability may contribute to reduced BS synthesis.

Another interesting finding of this study concerns the effects of EE on pool composition. A decreased contribution of cholate to the pool was found, as also previously reported by Kern *et al.* (5). Yet, synthesis of cholate was still appreciable in EE-treated intact rats. This is apparently in contrast to a previous study from our laboratory (10), in which we showed that the conversion of LDL-cholesterol to cholate is completely blocked in EE-treated rats. The combination of data implies that LDL-cholesterol is processed differently than cholesterol from other sources after EE administration. This may be a result of selective induction of LDL-receptors by EE in cell populations different from those expressing *CYP7A*, i.e. the periportal hepatocytes. Alternatively, it may be that in EE-treated rats LDL-cholesterol is preferably delivered to mitochondria for 27-hydroxylation rather than to the endoplasmic reticulum, where cholesterol 7α-hydroxylase resides.

The contribution of Δ^{22} β-muricholate to the pool was significantly increased in EE-treated rats. This newly identified rodent BS species is thought to represent a product of partial peroxisomal β-oxidation of the β-muricholate side chain (18,19,47). It is likely that the increased amounts of 6-hydroxylated BS species tentatively identified in bile of EE-treated rats by Kern *et al.* (5) actually represent Δ^{22} β-muricholate. Its increased contribution to the pool is probably the result of increased β-muricholate formation via the alternative pathway induced by EE: the fact that Δ^{22} β-muricholate disappears from the bile after interruption of the enterohepatic circulation is most likely explained by the assumption that β-muricholate can only be metabolized to Δ^{22} β-muricholate during entrohepatic cycling, i.e. after uptake from the intestine and transport back to the liver. In this scenario, therefore, Δ^{22} β-muricholate cannot be considered a primary BS but represents a tertiary species.

In conclusion, our studies show that EE treatment selectively suppresses the initial step in the neutral pathway of BS synthesis controlled by cholesterol 7α-hydroxylation in rats, at least in part at transcriptional level. EE does not affect sterol 27-hydroxylase activity and mRNA levels, probably leading to preferential BS synthesis via the alternative route. Yet, EE-effects on substrate availability and on enzymes further down the synthetic cascade may determine the ratios between the various end products of both synthetic pathways that are secreted into bile. This study therefore underlines

the importance of combining data on gene expression and enzyme activities with those on the actual metabolic fluxes.

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REFERENCES

1. Gumucio, J.J. and V.D. Valdivieso. Studies on the mechanism of the ethinylestradiol impairment of bile flow and bile salt secretion in the rat. Gastroenterology 1971;61:339-334
2. Bouchard, G., I.M. Yousef, and B. Tuchweber. Decreased biliary glutathione content is responsible for the decline in bile salt-independent flow induced by ethinyl estradiol in rats. Toxicol. Lett. 1994;74:221-233
3. Koopen, N.R., H. Wolters, R. Havinga, R.J. Vonk, P.L.M. Jansen, M. Müller, and F. Kuipers. Impaired activity of the bile canalicular organic anion transporter (mrp2/cmoat) is not the main cause of ethinylestradiol-induced cholestasis in the rat. Hepatology 1998;27:537-545
4. Davis, R.A. and F. Kern. Effects of ethinyl estradiol and phenobarbital on bile acid synthesis and biliary bile acid and cholesterol excretion. Gastroenterology 1976;70(6):1130-1135
5. Kern, F., H. Eriksson, T. Curstedt, and J. Sjövall. Effect of ethinylestradiol on biliary excretion of bile acids, phosphatidylcholines, and cholesterol in the bile fistula rat. J. Lipid. Res. 1977;18:623-634
6. Kirkpatrick, R.B. and P.G. Killenberg. Effects of ethinyl estradiol on enzymes catalyzing bile acid conjugation and sulfation. J. Lipid. Res. 1980;21:895-901
7. Bossard, R., B. Stieger, B. O'Neil, G. Fricker, and P.J. Meier. Ethinylestradiol treatment induces multiple canalicular membrane transport alterations in rat liver. J. Clin. Invest. 1993; 91:2714-2720
8. Simon, F.R., J. Fortune, M. Iwahashi, C. Gartung, A.W. Wolkoff, and E. Sutherland. Ethinyl estradiol cholestasis involves alterations in expression of liver sinusoidal transporters. Am. J. Physiol. 1996 ;271:G1043-G1052
9. Trauner, M., M. Arrese, C.L. Soroka, M. Ananthanarayanan, T.A. Koeppel, S.F. Schlosser, F.J. Suchy, D. Keppler, and J.L. Boyer. The rat canalicular conjugate export pump (mrp2) is down regulated in intrahepatic and obstructive cholestasis. Gastroenterology 1997 ;113:255-264
10. Kuipers, F., F.J. Nagelkerke, H. Bakkeren, R. Havinga, T.J.C. Van Berkel, and R.J. Vonk. Processing of cholesteryl ester from low-density lipoproteins in the rat. Hepatic metabolism and billiary secretion after uptake by different hepatic cell types. Biochem. J. 1989 ;257:699-704
11. Bertolotti, M. and D. Spady. Effect of hypocholesterolemic doses of 17a-ethinyl estradiol on cholesterol balance in liver and extrahepatic tissues. J. Lipid. Res. 1996;37:1812-1822
12. Chico, Y., O. Fresnedo, M. Lacort, and B. Ochoa. Effect of estradiol and progresterone on cholesterol 7a-hydroxylase activity in rats subjected to different feeding conditions. Steroids 1994 ;59:528-535
13. Axelson, M. and J. Sjövall. Potential bile acid precursors in plasma possible indicators of biosynthetic pathways to cholic and chenodeoxycholic acids in man. J Steroid Biochem 1990;36:631-640
14. Vlahcevic, Z.R., D.M. Heuman, and P.B. Hylemon. Regulation of bile acid synthesis. Hepatology 1991; 13:590-600
15. Princen, H.M.G., S.M. Post, and J. Twisk . Regulation of bile acid biosynthesis. Curr. Pharm. Design 1997;3:59-84
16. Vlahcevic, Z.R., T. Stravitz, D.M. Heuman, P.B. Hylemon, and W.M. Pandak. Quantitive estimations of the contribution of different bile acid pathways to total bile acid synthesis in the rat. Gastroenterology 1997; 113:1949-1957
17. Xu, G., G. Salen, S. Shefer, G.S. Tint, B.T. Kren, L.B. Nguyen, C.J. Steer, T.S. Chen, L. Salen, and D. Greenblat. Increased bile acid pool inhibits cholesterol 7a-hydroxylase in cholesterol fed rabbits. Gastroenterology 1997 ;113:1985-1965
18. Davis, R.A. and M.B. Thompson Nuclear magnetic resonance identification of the taurine conjugate of 3a,6b,7b,22 cholen-24-oic acid (tauro-d22-b-muricholate) in the serum of female rats treated with a-naphtylisothiocyanate. J. Lipid. Res. 1993;34:651-661
19. Setchell, K.D.R., F. Yamashita, C.M.P. Rodrigues, N.C. O'Connell, B.T. Kren, and C.J. Steer. d22-ursodeoxycholic acid, a unique metabolite of administered ursodeoxycholic acid in rats, indicating partial b-oxidation as a major pathway for bile acid metabolism. Biochemistry 1995;34:4169-4178
20. Semenkovich, C.F. and R.E. Ostlund. Estrogens induce low density lipoprotein receptor activity and decrease intracellular cholesterol in human hepatoma cell line Hep G2. Biochemistry 1987;26:4987-4992

21. Erikson, S.K., S. Jaeckle, S.R. Lear, S.M. Brady, and R.J. Havel. Regulation of hepatic cholesterol and lipoprotein metabolism in ethinyl estradiol-treated rats. J. Lipid. Res. 1989;30:1763-1771
22. Kern, F. and G.T. Eversson . Contraceptive steroids increase cholesterol in bile: mechanisms of action. J. Lipid. Res. 1987 ;28:828-839
23. Scheibner, J., M. Fuchs, M. Schieman, G. Tauber, E. Hormann, and E.F. Stange. Bile acid synthesis from newly synthesized vs preformed cholesterol precursor pools in the rat. Hepatology 1993; 17:1095-1102
24. Twisk, J., M.F.M. Hoekman, W.H. Mager, A.F.M. Moorman, P.A.J. de Boer, L. Sheja, H.M.G. Princen, and R. Gebhardt. Heterogeneous expression of cholesterol 7a-hydroxylase and sterol 27-hydroxylase genes in the rat liver lobulus. J. Clin. Invest. 1995; 95:1235-1243
25. Bandsma, R. H. J., Kuipers, F., Nagel, G. T., Elzinga, H., Boverhof, R., Neese, R. J., Hellerstein, M. K., and Stellaard, F. (1997) Clin. Nutr. 16, O55(Abstract)
26. Kuipers, F., R. Havinga, H. Bosschieter, G.P. Toorop, F.R. Hindriks, and R.J. Vonk. Enterohepatic circulation in the rat. Gastroenterology 1985;88:403-411
27. Murphy, G.M., B.H. Billing, and D.N. Baron. A fluorimetric and enzymatic method for the estimation of serum total bile acids. J. Clin. Pathol. 1970;23:594-598
28. Stellaard, F., M. Sackmann, T. Sauerbruch, and G. Paumgartner. Simultaneous determination of cholic acid and chenodeoxycholic acid pool sizes and fractional turnover rates in human serum using ^{13}C labeled bile acids. J. Lipid. Res. 1984;25:1313-1319
29. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randal. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1951;193:265-275
30. Chiang, J.Y.L. Reversed-phase high performance liquid chromatography assay of cholesterol 7 alpha hydroxylase. Methods Enzymol 1991; 206:483-491
31. Chomczynski, P. and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Ann. Biochem. 1987 ;162:156-158
32. Post, S.M., E.C.M. de Wit, and H.M.G. Princen. Cafestol, the cholesterol-raising factor in boiled coffee, suppresses bile acid synthesis by down regulation of cholesterol 7a-hydroxylase and sterol 27-hydroxylase in rat hepatocytes. Arterioscler. Thromb. Vasc. Biol. 1997 ;17:3064-3070
33. Eggertsen, G., M. Olin, U. Andersson, H. Ishida, S. Kubota, U. Hellman, K. Okuda, and I. Bjorkhem. Molecular cloning and expression of rabbit steol 12a-hydroxylase. J. Biol. Chem. 1996 ;211:32269-32275
34. Golder, W.N., B.A. Hollar, J.H. Waterson, and R.D. Schmickel. Molecular analysis of cloned human 18S ribosomal DNA fragments. Biochemistry 1978;75:5367-5371
35. Vonk, R.J., A.B. van Doorn, and J.H. Strubbe. Bile secretion and bile composition in the freely moving unanesthetized rat. Influence of food intake on bile flow. Clin. Sci. Mol. Med. 1978;55:253-259
36. Smit, M.J., A.M. Temmerman, R. Havinga, F. Kuipers, and R.J. Vonk . Short- and long-term effects of biliary drainage on hepatic cholesterol metabolism in the rat. Biochem. J. 1990;269:781-788
37. Windler, E.E.T., P.T. Kovanen, Y.S. Chao, M.S. Brown, R.J. Havel, and J.L. Goldstein. The estradiol-stimulated lipoprotein receptor of rat liver. J. Biol. Chem. 255(21): 1980;10464-10471
38. Staels, B., H. Jansen, A. van Tol, G. Stahnke, H. Will, G. Verhoeven, and J. Auwerx. Development, food intake, and ethinylestradiol influence hepatic triglyceride lipase and LDL receptor mRNA levels in rats. J. Lipid. Res. 1990 ;31:1211-1218
39. Adinofli, L.E., R. Utili, G.B. Gaeta, C.O. Abernathy, and H.J. Zimmerman. Cholestasis induced by estradiol-17-b-D-glucuronide: Mechanisms and prevention by sodium taurocholate. Hepatology 1984;4:30-37
40. Rosario, J., E. Sutherland, L. Zaccaro, and F.R. Simon. Ethinylestradiol administration selectively alters sinusoidal membrane lipid fluidity and protein composition. Biochemistry 1988;27:3939-3946
41. Kreek, M.J., R.E. Peterson, M.H. Sluisenger, and G.H. Jeffries. Effects of ethinylestradiol induced cholestasis on bile flow and biliary excretion of estradiol and estradiol glucuronide by the rat. Proc. Soc. Exp. Biol. Med. 1996;131:646-650
42. Bandsma, R.H.J., F. Stellaard, R.J. Vonk, G.T. Nagel, R.J. Neese, M.K. Hellerstein, and F. Kuipers. Contribution of newly synthesized cholesterol to rat plasma and bile by isotopomer distribution analysis: bile salt flux promotes secretion of newly synthesized cholesterol into bile. Biochem. J. 1998 ;329:699-703
43. Twisk, J., E.C.M. de Wit, and H.M.G. Princen. Suppresion of sterol 27-hydroxylase mRNA and transcriptional activity by bile acids in cultured rat hepatocytes. Biochem. J. 1995 ;305:505-511
44. Vlahcevic, Z.R., S.K. Jairath, D.M. Heuman, T. Stravitz, P.B. Hylemon, N.G. Avadhani, and W.M. Pandak. Transcriptional regulation of hepatic sterol 27-hydroxylase by bile acids. Am. J. Physiol. 1996 ;270:G646-G652
45. Araya, Z., H. Sjoberg, and K. Wikvall. Different effects on the expression of cyp7 and cyp27 in rabbit liver by cholic acid and cholestyramine. Biochem. Biophys. Res. Com. 1995;261:868-873

46. Duane, W.C., M.L. Gilberstadt, and G.M. Wiegand. Diurnal rythms of bile acid production in the rat. Am. J. Physiol. 1979;236:R175-R179
47. Rodrigues, C.M.P., B.T. Kren, C.J. Steer, and K.D.R. Setchell. Formation of d 22-bile acids in rats is not gender specific and occurs in the peroxisome. J. Lipid. Res. 1996 ;37:540-550

Chapter 4

Hepatic bile salt flux does not modulate level and activity of the sinusoidal Na^+-taurocholate cotransporter (ntcp) in rats

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ABSTRACT

Background/Aims: Efficient uptake at the basolateral plasma membrane of hepatocytes is required for maintenance of the enterohepatic circulation of bile salts. Uptake occurs mainly via a Na^+-dependent process mediated by ntcp, a recently cloned and characterized 51 kDa glycoprotein. The aim of this study was to evaluate the role of variations in hepatic bile salt flux through the liver in the regulation of ntcp activity and expression under non-cholestatic conditions.

Methods: We determined kinetics of Na^+-dependent taurocholate transport in isolated basolateral plasma membrane vesicles as well as hepatic ntcp protein and ntcp mRNA levels in long-term (8 days) bile-diverted rats, with a transhepatic BS flux of 0, and in streptozotocin-induced diabetic rats with a 2.5 fold increased BS flux.

Results: We found no changes in the kinetics of taurocholate transport in the absence of transhepatic BS flux due to bile diversion. Ntcp protein and ntcp mRNA levels were also unaffected in bile-diverted rats. Likewise, no changes in taurocholate transport kinetics, ntcp protein or ntcp mRNA levels were detected in streptozotocin-diabetic rats when compared to non-diabetic controls. Thus, variation of hepatic BS flux from 0 to 250% of normal values has no effect on hepatic ntcp expression or taurocholate transport activity inbasolateral plasma membrane vesicles in rats. In contrast, 4 days of bile duct ligation resulted in a strong decrease of ntcp mRNA and protein levels, as recently also reported by others.

Conclusion: Our data indicate that ntcp is not regulated by the flux across the sinusoidal membrane and /or intracellular concentrations of its physiological substrates under non-cholestatic conditions.

INTRODUCTION

Bile salts (BS) are maintained within an enterohepatic circulation to ensure optimal concentrations of these important biological detergents at the sites of their physiological actions, i.e. the hepatocyte, the bile canaliculus and the intestinal lumen. Proper functioning of BS transporting proteins in both liver and intestine is crucial for the maintenance of this enterohepatic circulation. The first step in the process of hepatic BS transport is uptake at the basolateral or sinusoidal membrane, which involves the action of the Na^+-taurocholate cotransporting protein (ntcp), a recently cloned 51 kDa glycoprotein (1). The sodium gradient required for this transport activity is generated by sinusoidal Na^+-K^+ ATPase activity (2,3). A number of studies has recently been published on the regulation of ntcp expression and transport activity *in vivo*, mainly focusing on cholestatic conditions by using experimental models such as bile duct ligation (4) or cytokine and endotoxin administration (5-7). The models of experimental cholestasis mentioned all lead to down-regulation of TC transport, ntcp protein density and mRNA levels. A detailed analysis of ontogenetic development of ntcp expression in relation to neonatal cholestasis has also been reported (8).

Limited data are available on regulation of this transporter under non-cholestatic conditions, in particular with respect to the question whether or not ntcp substrate availability plays a role in its regulation. Higgins and coworkers reported a down-regulation of Na^+-taurocholate co-transport activity in basolateral liver plasma membrane vesicles after 3 days of bile diversion, leading to exhaustion of the BS pool, in rats (9). No data on ntcp protein and mRNA levels were provided in this report. The presence of a putative BS regulatory element in the ntcp promoter was recently shown by Karpen *et al.* (10). This finding suggests a mode of regulation by BS at transcriptional level. On the other hand, it was shown in histochemical studies that ntcp is uniformly distributed across the liver acinus (11,12), whereas it is known that under physiological conditions BS are mainly transported through periportal cells (13). This suggests that expression of ntcp may be in fact independent of the BS flux. To address this apparent paradox, we have evaluated the role of transhepatic BS flux in the regulation of expression and activity of ntcp, by using two well-defined rat models. First, 8 days bile-diverted rats were used (14). Due to the absence of a circulating BS pool, there is no BS transport across the basolateral membrane in these animals. All biliary BS are derived from hepatic *de novo* synthesis under these conditions. As a consequence, these rats have an effective transhepatic BS flux of zero. Second, streptozotocin-induced diabetes was used as a model with increased BS flux. It is well established that long term streptozotocin-diabetes in rats leads to an increased BS pool size (15,16) and an increased maximal secretory rate (S_{RM}) for BS (15). These phenomena can be completely normalized by insulin administration. The mechanisms underlying this diabetes-specific increase of BS pool size and secretion rate are still not well understood. In our hands, BS flux in the diabetes model is about 250% of control values. In the bile diversion and diabetes models, we determined sodium-dependent TC transport in hepatic basolateral membrane vesicles, plasma membrane ntcp protein

levels by Western analysis and hepatic ntcp mRNA levels by Northern blotting. For comparison, a group of bile duct ligated rats was included in this study, since it has recently been shown that bile duct ligation leads to a rapid down-regulation of ntcp protein and mRNA levels in rats (4).

MATERIALS AND METHODS

Chemicals. [^{3}H(G)]-taurocholate (3.47 Ci/mmol; 99% pure by HPLC and thin layer chromatography) was obtained from Du Pont/New England Nuclear (Boston, MA). Unlabeled taurocholate (TC) was obtained from Calbiochem (La Jolla, CA). Streptozotocin was purchased from Upjohn (Kalamazoo, MI). All other chemicals were of reagent grade or the highest purity grade commercially available.

Animals. Male Wistar rats (Harlan Laboratories, Zeist, The Netherlands) weighing 230-340 g were housed in an environmentally controlled facility with diurnal light cycling and free access to food and water. Experimental protocols were approved by the Ethical Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen.

Chronic bile diversion. Animals were equipped with a permanent silastic catheter in the bile duct, as described by Kuipers et al. (14). Control animals were sham-operated by performing laparotomy and manipulation without cannulation of the bile duct. Bile-diverted and sham-operated animals were housed in individual cages. Animals were allowed to drink 0.9% NaCl to avoid hyponatremic conditions due to loss of electrolytes via bile. Bile was diverted for 8 days; after this period the body weight had returned to pre-operative values. Animals were killed by decapitation between 9.00 and 10.00 am.

Induction of diabetes. Diabetes was induced by a single intraperitonal injection of steptozotocin (6 mg /100g body weight). The control group was given a saline injection. Animals were checked for urinary glucose using glucose sticks (Boehringer Mannheim, Mannheim, Germany). Animals were killed 4 weeks after induction of diabetes by decapitation. Separate groups of diabetic and control rats were equipped with permanent catheters in bile duct and duodenum (14), about 3 weeks after streptozotocin injection. Both catheters were immediately connected to maintain an intact enterohepatic circulation. Eight days after surgery, i.e. after complete recovery, both catheters were disconnected and bile was collected continuously for 5 hours in 0.5 h intervals by means of a fraction collector. BS concentrations were determined by an enzymatic fluorometric assay (14) and used to calculate BS output rates. BS pool size and bile salt synthesis were determined from the wash-out curve as described (14). BS concentrations in plasma were also determined enzymatically.

Bile duct ligation. The bile duct was doubly-ligated close to the liver hilus immediately below the bifurcation and cut between the ligatures. Control animals were sham-operated by laparotomy and

manipulation of the bile duct without ligation of the duct. Ligated and sham-operated animals were housed in separate cages. Animals were killed by decapitation 4 days after surgery.

Specimen collection. After decapitation, blood was collected in EDTA-containing tubes. Small sections of liver material were removed and quickly frozen in liquid N_2 for RNA isolation. Subsequently, the remainder of the livers was harvested and weighed.

Preparation of rat liver basolateral plasma membrane vesicles (blLPM) and determination of marker enzyme activities and protein concentrations. Rat liver basolateral membrane vesicles were prepared essentially as described by Meier et al. (17), with minor modifications described by Wolters et al. (18). The final membrane pellet was suspended in buffer containing 300 mM sucrose, 0.2 mM $CaCl_2$, 10 mM $MgSO_4$, 10 mM HEPES pH 7.5. The membrane aliquots were frozen and stored until use in liquid nitrogen.

Protein concentrations were determined according to Lowry et al. (19). Relative enrichment of Na^+-K^+-ATPase as marker enzyme for the basolateral fraction and Mg^{2+}ATPase as marker enzyme for the canalicular fraction, i.e. the activity of the enzyme in the isolated plasma membrane preparation divided by the activity in the homogenate, was used to determine the degree of purification of the isolated membranes in the different experimental groups. Na^+-K^+ ATPase and Mg^{2+}ATPase activity were measured according to Scharschmidt et al.(20) using a Uvikon 931 spectrophotometer (Kontron Instruments, Milan, Italy).

Transport studies. Transport studies were carried out in blLPM vesicles using a rapid filtration technique (21). Five μl membrane vesicles (15 μg protein) were preincubated at 25°C for 1 min. Uptake was initiated by addition of 20 μl prewarmed incubation medium (final concentration: 100 mM NaCl or KCl, 100 mM sucrose, 10 mM HEPES pH 7.5, 0.2 mM CaCl and 10 mM $MgSO_4$, BSA 1 mg/ml , [^{3}H]-TC was added in different concentrations) to the membranes. Uptake was performed at 25°C. Uptake was stopped by adding 750 μl of ice cold stop solution (100 mM sucrose, 100 mM KCl, 10 mM HEPES pH 7.5, 0.2 mM $CaCl_2$ and 10 mM $MgSO_4$) to the incubation medium. The sample was immediately filtered through a 0.45 μm Millipore filter (Millipore, Bedford, MA) that was prewashed with 1 ml stop solution containing 1 mM unlabeled TC, and then washed twice with 4 ml ice cold stop solution. The filters were dissolved in Ultima gold MV scintillation fluid (Packard Instruments, Dowers Grove, IL) and counted in a liquid scintillation counter type Packard 1500 (Packard Instruments, Dowers Grove, IL).

Western Blot Analysis. Liver tissue was homogenized in 10 volumes 1 mM $NaHCO_3$ pH 7.5 with 17 mg/L phenylmethylsulfonylfluoride (PMSF). The homogenate was centrifuged for one hour at 100.000 g and 4°C. The pellet was resuspended in 10 mM Tris-HCl pH 7.5, 250 mM sucrose with protease

inhibitor cocktail Complete ™(Boehringer Mannheim, Mannheim, Germany, 1 tablet/50 ml). Protein concentrations were determined according to Lowry (19). Seventy-five μg of protein was separated using SDS gel electrophoresis, transferred to nitrocellulose membrane (Amersham, Little Chalfont, UK), and probed with anti -ntcp- immunoglobulin(Ig)G K4 (12). Immune complexes were detected using horseradish peroxidase-conjugated donkey anti rabbit IgG by the ECL Western blotting kit (Amersham, Little Chalfont, UK).

Northern Blot Analysis. Total RNA was isolated using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and chloroform-phenol extraction (22), subsequently separated on agarose formaldehyde gel and transferred to a nylon membrane, Hybond N (Amersham, Little Chalfont, UK), by overnight blotting. Blots were prehybridized in standard hybridizing solution (0.5 M $NaHPO_4$, 1% crystaline grade BSA, 1 mM Na_2EDTA and 7 % SDS, pH 7.2 and 100 μg herring sperm DNA per ml). Blots were hybridized at 65°C overnight at 1-2 $*10^6$ cpm/ml in hybridization solution. Blots were washed in washing buffer (40 mM $NaHPO_4$, 1 mM Na_2EDTA and 1% SDS pH 7.2). cDNA probes were labeled using a random primed labeling kit (Boehringer Mannheim, Mannheim, Germany) to a specific activity of 10^8-10^9 cpm/μg. Activities were corrected for concentration differences, using 28S rRNA as a probe.

Statistics. Statistical analysis between the experimental groups was performed using the Student's *t*-test. Statistical significance was considered at p values of < 0.05. Data represent the mean $\pm$ SD of 4-5 animals per group at each time point.

RESULTS

Animal characteristics. After 8 days of bile diversion there was no significant difference in body weight between sham-operated and bile-diverted rats, as shown in Table 1. Both groups gained about 10 grams compared with preoperative body weight during the 8-day period. Saline-injected control rats gained significantly more weight than the streptozotocin-diabetic rats during 4 weeks, as previously reported (23). Liver weights in the sham and bile-diverted groups were not different, but the diabetic group had a significantly lower liver weight than the saline-injected controls. The liver weight as % of body weight did not differ between these groups. All diabetic animals included in this study were found positive for urinary glucose.

	n	Body weight after (g)	Liver weight (g)	Liver (% body weight)
sham operation	15	321±30	11.9±1.9	3.71±0.30
bile diversion	14	314±26	11.7±1.6	3.71±0.25
saline injection	18	369±19	15.3±1.5	4.12±0.30
strepto-diabetes	16	288±27*	13.7±1.4*	4.78±0.25

*Values are mean ± SD. No significant differences were found between sham-operated and bile-diverted rats. Diabetic rats had significantly lower body- and liver weight at 4 weeks after streptozotocin injection. *p<0.05. Liver weight as % of the body weight was not significantly different between these groups.*

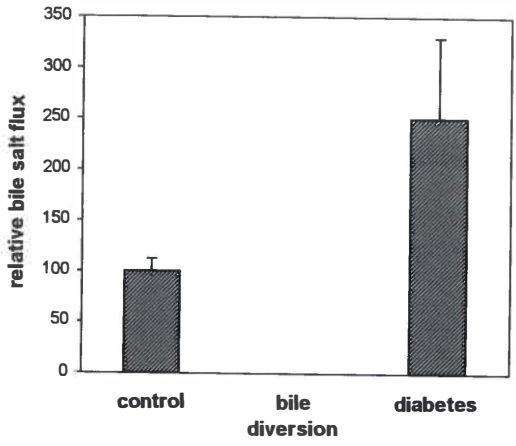
Table1. Body weight after experiment and liver weight in sham-operated, bile-diverted, saline-injected control and diabetic rats.

	plasma bile salt (μM)
sham operation	22.6 ± 10.6
bile diversion	n.d
saline injection	27.3 ± 9.0
strepto-diabetes	110.0 ± 37.1*

*Values are means ± SD. Bile-diverted rats had bile salt concentrations that were below detection limits (n.d., not detectable). Diabetic rats had significantly higher plasma bile salt concentrations at 4 weeks after streptozotocin injection than in saline-injected controls. *p<0.05.*

Table 2. Plasma bile salt concentrations in sham-operated, bile-diverted, saline-injected control and diabetic rats.

Bile salt flux. To asses the physiological transhepatic BS flux in diabetic and control rats, BS output rates during a 6-h period following interruption of the enterohepatic circulation were determined (Figure 1a). These values are derived from studies with unanaesthesized rats equipped with permanent catheters in bile duct and duodenum, to exclude potential interference of anesthetics and stress restraint. The output rates shown represent secretion of BS originating from the circulating pool and from *de novo* synthesis. BS pool sizes as calculated from the wash out curves, were 56.6 ± 12.3 μmol /100g in the controls and 148.2 ± 49.5 μmol /100g in diabetic animals (plus 162%). Bile salt *de novo* synthesis as determined from these curves was 1.73 ± 0.54 μmol/100g in the controls and 2.57 ± 0.66 μmol/100g in the diabetic animals (plus 49%). The higher BS output in diabetic animals resulted in an increased



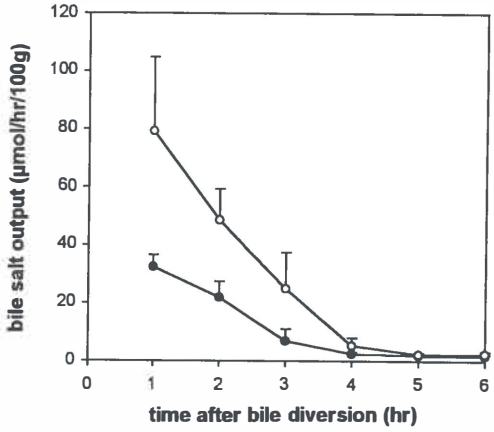


Figure 1a. BS output rates during 6 hours after interuption of the enterohepatic circulation at time zero in saline-injected control (-●-) and streptozotocin-diabetic rats (-○-). *The bile salt synthesis was determined from the output after exhaustion of the BS pool i.e. the average BS output after 5 hours of bile diversion. Mean values ±SD are shown for 5 rats per group.*

Figure 1b. Relative BS flux was calculated by subtracting the BS synthesis rate from the output rate during the first hour. *This figure represents relative BS flux in saline-injected control, bile-diverted rats and streptozotocin-diabetic rats. Mean values ± SD are given for 5 rats per group. Bile salt flux is zero by definition in bile-diverted rats.*

calculated transhepatic BS flux in these animals, as shown in Figure 1b. This flux represents the BS output during the first hour after initiation of the bile collection, i.e., the physiological output rate, minus the synthesis rate determined after exhaustion of the BS pool (14).

In contrast, BS output in long-term bile-diverted rats represents *de novo* synthesis only. The transhepatic BS flux in bile-diverted animals, by definition, is zero (Figure 1b).

Table 2 shows plasma BS concentrations in the four groups. As shown BS concentration in plasma of steptozotocin-diabetic rats was 4-fold increased compared with their controls. Plasma BS concentrations in bile-diverted rats were below a level that allows reliable quantitation by our enzymatic assay.

Membrane enrichment. Enrichments of the Na^+-K^+ ATPase activity in blLPM fractions relative to liver homogenates in the four experimental groups are summarized in Table 3; similar average values were found across all groups, with individual variations ranging from a factor 12 to a factor 42. These fractions were slightly contaminated with canalicular membranes as shown by the relative enrichment in Mg^{2+}ATPase activity. NADPH-cytochrome *c* reductase activity as marker for contamination with endoplasmic reticulum was comparable to that of earlier reported membrane preparations (18). Absolute Na^+ -K^+ ATPase activities in homogenates of diabetic livers were significantly higher than in control livers, as reported earlier (23). Bile diversion had no effect on total hepatic Na^+-K^+ ATPase activity.

	Na^+-K^+ATPase activity in homogenates µmol/mg protein/hr	*Relative enrichment of Na^+-K^+ATPase activity in blLPM fraction*	*Relative enrichment of Mg^+ATPase activity in blLPM fraction*
sham operation	1.14±0.25	28±13	7±0.9
bile diversion	1.01±0.07	22±3	6±2.1
saline injection	1.07±0.16	17±5	6±1.7
strepto-diabetes	1.66±0.16*	21±5	7±0.8

Values are mean ± SD. 4-5 preparations per group.
** $p<0.05$.*

Table 3 Na^+-K^+ATPase activity in homogenates and relative enrichment of Na^+-K^+ATPase activity and Mg^+ATPase activity in blLPM of sham-operated, bile-diverted, saline-injected control and diabetic rats.

Taurocholate uptake in isolated blLPM. Km and Vmax values of TC uptake were determined by measuring the initial uptake velocity over a concentration range between 7 and 200 µM. These velocities were measured in the presence of an inwardly directed Na^+ or K^+ gradient: the difference between these two measurements represents Na^+-dependent, ntcp-mediated transport. Both the Km and Vmax values were not significantly different between the experimental groups, as summarized in

Table 4. When corrected for the enrichment in the Na^+-K^+ ATPase activity, Vmax values were virtually identical across all four groups (Table 4).

	Km (*µmol/l*)	*Vmax* (*nmol/min/mg protein*)	*Vmax / rel. enrichment*
sham operation	16±2	4.95±1.97	0.20±0.11
bile diversion	18±3	3.27±1.17	0.15±0.04
saline injection	26±7	2.76±1.25	0.18±0.11
strepto-diabetes	24±9	3.88±1.26	0.20±0.12

Values are mean ± SD. 4-5 preparations per group

Table 4. Kinetic parameters of sodium dependent taurocholate transport in blLPM of sham-operated, bile-diverted, saline-injected control and diabetic rats and the ratio between Vmax and relative enrichment in Na^+-K^+ ATPase activity.

Protein expression. For Western blotting, we used total liver membrane fractions instead of blLPM vesicles to overcome the differences in enrichment between different preparations. Representative results are shown in Figure 2. During short exposure of the films we consequently found a double band pattern for the ntcp protein, that is not visible after longer exposure. This pattern could be due to a partial deglycosylation of the protein (12). Ntcp protein levels did not change after 8 days of bile diversion or in diabetes. In contrast, in rats with 4 days bile duct ligation ntcp protein was almost not detectable (Figure 3), confirming previous data from Gartung *et al.* (4).

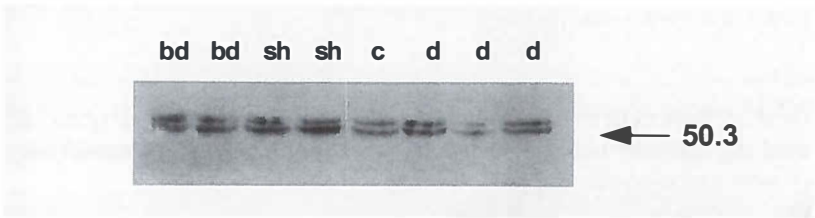


Figure 2. Ntcp protein mass in total hepatic membrane fractions of bile-diverted (bd), sham-operated (sh), saline-injected control (c) and streptozotocin-diabetic (d) rats. *A representative Western blot is shown.*

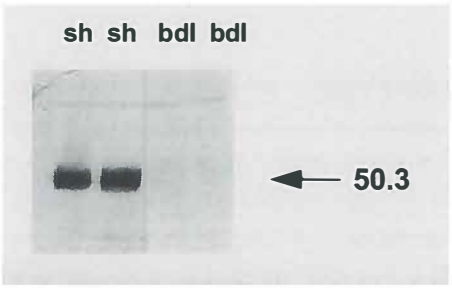


Figure 3. Ntcp protein mass in total hepatic membrane fractions of sham-operated control rats (sh) and of rats after 4 days of bile duct ligation (bdl). *A representative Western blot is shown.*

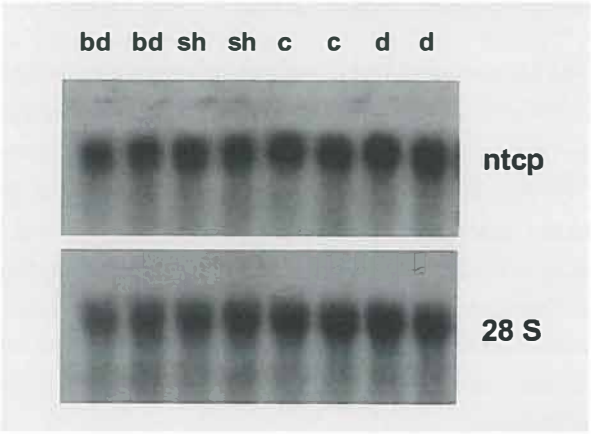


Figure 4. Hepatic ntcp mRNA levels and 28S rRNA levels in sham-operated (sh), bile-diverted (bd), saline-injected control (c) and streptozotocin-diabetic rats (d).

Steady-state mRNA levels. Steady-state mRNA levels were quantified using Northern blot analysis. To exclude effects of differential RNA loading, the 28S rRNA levels were determined for normalization. This ntcp / 28S ratio was about 1 in al groups as shown in figure 4, indicating no differences between the groups in this respect.

DISCUSSION

In the present study, we investigated whether or not changes in transhepatic BS flux play a role in the regulation of ntcp under non-cholestatic conditions in rats. We used two well-established models with wide variations in BS flux. The first model, developed and characterized by Kuipers and coworkers is the unrestrained bile-diverted rat (14). Bile was diverted for an 8-day period, because we have shown previously that this period of time allows for a complete recovery from the surgical procedure with respect to food intake and body weight (14). After 8 days of bile diversion, hepatic BS synthesis is maximally upregulated and no BS can be detected in portal and peripheral blood due to exhaustion of the circulating BS pool (14). In this situation, all biliary BS are derived from hepatic *de novo* synthesis and the effective BS flux across the sinusoidal liver plasma membrane is zero.

An increased hepatic BS flux was established by induction of streptozotocin-diabetes. Villanueva *et al.* (23) have shown that BS pool size, BS output as well as biliary lipid secretion is increased in this model. This effect can be completely reversed by insulin administration, showing that it is a specific diabetes effect (23). BS pool size and lipid secretion are also increased in humans with diabetes (16,24). The mechanisms underlying these effects of diabetes are not well understood. Insulin-deficiency could lead to increased gene transcription of cholesterol 7α-hydroxylase and sterol 27-hydroxylase and as a consequence of a increased BS synthesis (25). We found that BS synthesis is indeed slightly increased in diabetic rats, but to a much lesser extent than the increase in BS poolsize. It may be that the intestinal conservation of BS is more effective in the diabetic state.

To avoid potential toxic effects of streptozotocin in our experiments (16) and to allow a maximal increase in the BS pool, animals were studied 4 weeks after injection of the drug. In our hands, BS pool size increased by 162% and the calculated transhepatic flux by 150 %. These values are in good agreement with earlier published data (15,23).
The results of our study indicate that large fluctuations in BS flux under non-cholestatic conditions do not regulate ntcp transport activity with respect to affinity and carrier density, as is evident from unaffected Km and Vmax values. The small, non-significant differences that we found between experimental groups could largely be explained by differences in enrichment of the isolated blLPM fractions. Differences in enrichment values between separate isolations represent a commonly encountered phenomenon when performing transport studies with isolated liver plasma membrane vesicles. These differences were irrespective of the experimental groups and, therefore, Vmax values could be corrected for these enrichment differences.

Protein level was studied by Western blot analysis and confirmed the transport results: no differences between bile-diverted animals, diabetic rats and their respective controls were found. On the other hand, ntcp levels in bile duct-ligated rats drastically declined within 4 days, as also shown recently by

Gartung et al. (4). Evaluation of mRNA levels in our models revealed that, indeed, the expression of *ntcp* was not altered in our experimental models in contrast to the bile duct ligated rats.

Our data on the effects of bile diversion are in contrast with those of Higgins et al. (9). These authors recently published data to suggest that bile diversion in rats leads to down-regulation of Na^+-dependent TC transport in blLPM vesicles. A possible explanation for the difference between our results and those of Higgins and co-workers may be related to the duration of bile diversion. We used 8 days of bile diversion because this time interval allows the animals to recover completely from the surgical procedure, as shown by increased food intake and unaffected body weight. It is possible that the down-regulation in ntcp transport activity after 3 days of bile diversion is related to consequences of anesthesia and surgical stress. In the study mentioned the rats lost about 13 % of their body weight. In contrast to our study, Higgins et al. show data on transport in isolated blLPM only; no data on protein and mRNA levels were presented.

The fact that ntcp is homogeneously distributed within the liver acinus in normal rats, as shown by Stieger et al. (12) and Ananthanarayanan et al. (11), and confirmed in our laboratory (data not shown), is consistent with our finding that physiological differences in BS flux do not influence *ntcp* expression, even if the BS flux at the sinusoidal membrane is reduced to zero. Indeed, kinetic and autoradiographic studies by Groothuis et al. (13) have shown that there is a steep BS gradient along the acinus and that mainly the periportal cells are active in BS transport under physiological conditions. As ntcp is present equally in all cells of the liver acinus, this strongly indicates that ntcp is constitutively expressed and that physiological fluctuations do not influence this expression. During extreme high BS load at the sinusoidal membrane, probably other bile salt transporting mechanisms, including the organic anion transporting proteins (oatp), with lower affinity for BS than ntcp could serve as additional BS transporting systems (26).

Gartung et al. (4) convincingly showed that under cholestatic conditions ntcp expression is down-regulated as is also the case after partial hepatectomy (27). Endotoxin and TNFα administration also reduce ntcp transport activity, protein levels and mRNA (5-7). Furthermore it is known that the ntcp expression disappears within 72 hr in cultured hepatocytes (28). Ntcp appears late during gestation, reaching adult levels about 3 weeks after birth (8). Sofar, prolactin is the only factor known to be able to upregulate ntcp expression (29). Prolactin is thus probably one of the factors that is responsible for the induction of ntcp during gestation.

A common phenomenon in ntcp down-regulated systems is the loss of functional secretory polarity (i.e. cholestasis) or differentiation of the hepatocytes. The question that remains concerns the nature of the specific regulators of the ntcp under these conditions. Potential candidates are specific cytokines (5-7,30). BS also remain candidates because in cholestatic states intracellular BS

concentrations are considerably increased. A 7-8 fold increase in liver tissue BS concentration after bile duct ligation was recently reported by Setchell et al. (31). In this respect it is important to note that Karpen et al. (10,32) recently found a putative BS binding element in the ntcp promoter. In case of cholestasis with high intracellular concentrations of BS, BS binding to the ntcp promoter region may represent a key step in ntcp down-regulation, but these intracellular BS concentrations must obviously exceed the concentrations that are reached in streptozotocin-diabetes. It may be that a combination of BS together with other cholestasis-related factors is important for down-regulation of ntcp expression.

In summary, the present study shows that the ntcp transport activity, ntcp protein levels and mRNA levels are not affected by "physiological fluctuations" in transhepatic BS flux. These data suggest that, under normal conditions, ntcp is constitutively expressed and that ntcp is down-regulated only under more stringent pathological conditions, like bile duct ligation, partial hepatectomy and cell culture. The specific factors that affect the ntcp gene expression under these pathological conditions remain to be identified.

Acknowledgements

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REFERENCES

1. Hagenbuch B, Stieger B, Foguet M, Lübbert H, Meier PJ. Functional expression cloning and characterization of the hepatocyte Na+/bile acid cotransport system. Proc Natl Acad Sci 1991;88:10629-10633.
2. Scharschmidt BF, Stephens JE. Transport of sodium, chloride and taurocholate by cultured rat hepatocytes. Proc Natl Acad Sci 1981;78:986-990.
3. Swartz LRR, Burr M, Schwenk M, Pfatt M, Greim H. Uptake of taurocholic acid into isolated rat liver cells. Eur J Biochem 1975;55:617-623.
4. Gartung C, Ananthanarayanan M, Rahman M, Schuele S, Nundy S, Soroka CL, Stolz A, Suchy FL, Boyer JL. Down-regulation of expression and function of the rat liver Na/Bile acid cotransporter in extrahepatic cholestasis. Gastroenterology 1996;110:199-209.
5. Green RM, Whiting JF, Rosenbluth AB, Beier D, Gollan JL. Interleukin-6 inhibits hepatocyte taurocholate uptake and sodium-potassium-adenosinetriphosphatase activity. Am J Physiol 1994;267:G1094-G1100.
6. Moseley RH, Wang W, Takeda H, Lown K, Shick L, Ananthanarayanan M, Suchy FJ. Effect of endotoxin on bile transport in rat liver: a potential model for sepsis-associated cholestasis. Am J Physiol 1996;271:G137-G146.
7. Trauner M, Gartung C, Schlosser SF, Boyer JL. Role of nitric oxide in the transcriptional regulation of bile acid transporters in endotoxin induced cholestasis. Gastroenterology 1996;104:a1349(Abstract).
8. Boyer JL, Hagenbuch B, Ananthanarayanan M, Suchy F, Stieger B, Meier PJ. Phylogenic and ontogenic expression of hepatocellular bile acid transport. Proc Natl Acad Sci 1993;90:435-438.
9. Higgins JV, Paul JM, Dumaswala R, Heubi JE. Downregulation of taurocholate transport by ileal BBM and liver BLM in biliary diverted rats. Am J Physiol 1994;267:G501-G507.
10. Karpen SJ, Ananthanarayanan M, Potselueva T, Suchy FJ. Bile acids suppres the rat liver Na^+-dependent bile acid cotransporter gene promoter. Hepatology 1995;22:318a(Abstract).

11. Ananthanarayanan M, Cheng OI, Boyer JL, Suchy FJ. Characterization of cloned rat liver Na+-bile acid cotransporter using peptide and fusion protein antibodies. Am J Physiol 1994;267:G637-G643.
12. Stieger B, Hagenbuch B, Landmann L, Höchli M, Schroeder A, Meier PJ. In situ localization of the hepatocytic Na+/ Taurocholate cotransporting polypeptide in rat liver. Gastroenterology 1994;107:1781-1787.
13. Groothuis GMM, Hardonk MJ, Keulemans KPT, Nieuwenhuis P, Meijer DFK. Autoradiographic and kinetic demonstration of acinar hetrogenity of taurocholate transport. Am J Physiol 1982;243:G455-G462.
14. Kuipers F, Havinga R, Bosschieter H, Toorop GP, Hindriks FR, Vonk RJ. Enterohepatic circulation in the rat. Gastroenterology 1985;88:403-411.
15. Icarte MA, Pizarro M, Accatino L. Adaptive regulation of hepatic bile salt transport: effects of alloxan diabetes in the rat. Hepatology 1991;14:671-678.
16. Watkins JB, Sanders RA. Diabetes Mellitus-induced alterations of hepatobiliary function. Pharmacol Rev 1995;47:1-23.
17. Meier PJ, Sztul ES, Reuben A, Boyer JL. Structural and functional polarity of canalicular and basolateral plasma membrane vesicles isolated in high yield from rat liver. J Cell Biol 1984;98:991-1000.
18. Wolters H, Spiering M, Gerding A, Slooff MJH, Kuipers F, Hardonk M, Vonk RJ. Isolation and characterization of canalicular and basolateral plasma membrane fractions from human liver. Biochim Biophys Acta 1991;1069:61-69.
19. Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-275.
20. Scharschmidt BF, Keeffe EB, Blankenship NM, Ockner RK. Validation of a recording spectrophotometric method for measurement of membrane-associated Mg- and NaK-ATPase activity. J Lab Clin Med 1979;93:790-799.
21. Meier PJ, Meier-Abt AS, Barret C, Boyer JL. Mechanisms of taurocholate transport in canalicular and basolateral rat liver plasma membranes. J Biol Chem 1984;259:10614-10622.
22. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning. Cold Spring Harbor,N.T. Cold Spring Harbor Laboratory, 1989:188-209.
23. Villanueva GR, Herreros M, Perez-Barriocanal F, Bolanos JP, Marin JJG. Enhancement of bile acid-induced biliary lipid secretion by streptozotocin in rats: Role of insulin deficiency. J Lab Clin Med 1990;115:441-448.
24. Feldman M, Feldman M, Jr. The incidence of cholelithiasis, cholesterosis and liver disease in diabetes mellitus. An autopsy study. Diabetes 1954;3:305-307.
25. Twisk J, Hoekman MFM, Lehmann EM, Meijer P, Mager WH, Princen HG. Insulin suppresses bile acid synthesis in cultured rat hepatocytes by down regulation of cholesterol 7a-hydroxylase and sterol 27-hydroxylase gene transcription. Hepatology 1995;21:501-510.
26. Kullack-Ublick GA, Hagenbuch B, Stieger B, Wolkoff AW, Meier PJ. Functional characterization of basolateral rat liver organic anion transporting polypeptide. Hepatology 1994;20:411-416.
27. Green RM, Lipin AL, Pelletier EM, Hagenbuch B, Meier PJ, Beier D, Gollan JL. Hepatic regeneration is associated with a marked reduction in mRNA expression of the basolateral Na^+-taurocholate transporter. Gastroenterology 1994;106:A901(Abstract).
28. Liang D, Hagenbuch B, Stieger B, Meier PJ. Parallel decrease of Na^+-taurocholate cotransport and its encoding mRNA in primary cultures of rat hepatocytes. Hepatology 1993;18:1162-1166.
29. Liu L, Ganguly T, Hyde JF, Vore M. Prolactin increases mRNAencoding Na+-TC cotransport polypeptide and hepatic Na+-TC cotransport. Am J Physiol 1995;268:G11-G17.
30. Green RM, Beier D, Gollan JL. Regulation of hepatocyte bile salt transporters by endotoxin and inflammatory cytokines in rodents. Gastroenterology 1996;111:193-198.
31. Setchell KDR, Rodrigues CMP, Clerici C, Solinas A, Morelli A, Gartung C, Boyer JL. Bile acid concentrations in human and rat liver tissue and hepatocyte nuclei. Gastroenterology 1997;112:226-235.
32. Karpen SJ, Sun AQ, Kudish B, Hagenbuch B, Meier PJ, Ananthanarayanan M, Suchy FJ. Multiple factors regulate the rat liver basolateral sodium dependent bile acid cotransporter gene promoter. J Biol Chem 1996;271:15211-15221.

Chapter 5

Decreased Na^+-dependent taurocholate uptake and low expression of the sinusoidal Na^+-taurocholate cotransporting protein (ntcp) in livers of mdr2 p-glycoprotein-deficient mice.

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Submitted

ABSTRACT

Background / Aims: Ntcp-mediated uptake of bile salts at the basolateral membrane of hepatocytes is required for maintenance of their enterohepatic circulation. Expression of Ntcp is reduced in various experimental models of cholestasis associated with increased plasma bile salt concentrations. Mdr2 P-glycoprotein-deficient mice lack biliary phospholipids and cholesterol but show unchanged biliary bile salt secretion and increased bile flow. These mice are evidently not cholestatic, but plasma bile salt concentrations are markedly increased. The aim of this study was to investigate the role of Ntcp in the elevated bile salt levels in mdr2 P-glycoprotein-deficient (-/-) mice.

Methods: Plasma membranes were isolated from male wild type (+/+) and *mdr2* (-/-) mice for measurement of Na^+-dependent taurocholate transport and assessment of Ntcp protein levels by Western blotting. Northern blot analysis and competitive RT-PCR were used to determine hepatic *Ntcp* mRNA levels.

Results: Kinetic analysis showed a 2-fold decrease in the V_{max} of Na^+-dependent taurocholate transport with an unaffected K_m in (-/-) mice compared with (+/+) controls. Ntcp protein levels were 4-6 fold reduced in plasma membranes of (-/-) mice relative to sex-matched controls. Surprisingly, hepatic *Ntcp* mRNA levels were not significantly affected in the (-/-) mice.

Conclusions: Elevated plasma bile salt levels in mdr2 P-glycoprotein-deficient mice in the absence of overt cholestasis are associated with reduced Ntcp expression and transport activity. This is due to posttranscriptional down-regulation of Ntcp.

INTRODUCTION

Uptake of bile salts from portal blood at the sinusoidal membrane of hepatocytes predominantly takes place by a sodium-dependent process mediated by Na^+-taurocholate cotransporting protein (Ntcp). Ntcp-mediated uptake is thought to be essential for maintaining the enterohepatic circulation of bile salts. The expression and function of Ntcp is not regulated by physiological fluctuations in hepatic bile salt flux (1), but is down-regulated in various models of experimental cholestasis, including cholestasis induced by endotoxin or TNFα administration, bile duct ligation and ethinylestradiol administration (2-6). Partial hepatectomy also leads to rapid down-regulation of Ntcp in rats (7). Cholestatic conditions are usually associated with increased plasma bile salts levels: Ntcp expression appears to be inversely related to plasma bile salt levels, probably reflecting their intracellular concentrations, in cholestatic rat models (8).

Mice in which the gene encoding for mdr2 P-glycoprotein (Pgp) has been disrupted ((-/-) mice) are unable to secrete phospholipids and cholesterol into bile, but show increased bile flow and an unchanged biliary bile salt secretion (9). These mice are evidently not cholestatic when cholestasis is defined as "impairment or cessation of bile flow" (10-12). Yet, these mice have elevated plasma bile salt and bilirubin levels. Furthermore, plasma levels of alkaline phosphatase, aspartate transaminase and alanine transaminase are increased when compared with (+/-) and (+/+) control mice, indicating damage of liver parenchyma. Morphologically, the livers of *mdr2* knockout mice show some degeneration of hepatocytes, ductular proliferation and portal inflammation. Liver pathology in these mice has been shown to be related to the formation of lipid-free bile, exerting cytotoxic actions of bile salts towards bile duct epithelial cells (13,14).

To provide a mechanistic basis for the elevated plasma bile salt levels in the absence of cholestasis in mdr2 Pgp-deficient mice, we determined the Na^+-dependent transport of taurocholate in plasma membrane vesicles isolated from livers of these mice and we examined hepatic Ntcp protein and *Ntcp* mRNA levels.

MATERIALS AND METHODS

Chemicals. [^{3}H(G)]-taurocholate (3.47 Ci/mmol; 99% pure by HPLC and thin layer chromatography) was obtained from Du Pont/New England Nuclear (Boston, MA, USA). Unlabeled taurocholate was obtained from Calbiochem (La Jolla, CA, USA). The polyclonal Ntcp antibody (K4) and the complementary DNA used were described previously (1,15,16). All other chemicals were of reagent grade or the highest purity grade commercially available and purchased from Sigma Chemicals (St. Louis, MO, USA) or Amersham (Little Chalfont, UK).

Animals. Homozygous *mdr2* (-/-) mice and control (+/+) mice of the FVB strain (17) used in these experiments were bred at the animal laboratory of the University of Amsterdam. Animals were used at 3-6 months of age and were kept in a light- and temperature-controlled environment. The mice had free access to lab chow and tapwater prior to the experiments. The animals received humane care and experimental protocols complied with the local guidelines for use of experimental animals.

Prior to removal of the liver, the mice were anaesthetized with halothane and a blood sample was obtained by cardiac punction. The liver was weighed and immediately transferred to ice-cold $NaHCO_3$ buffer (see below). A small part of the liver was removed at this point and frozen quickly in liquid nitrogen for subsequent RNA isolation.

Analyses. Bile salts in plasma were determined by an enzymatic fluorimetric assay (18). Aspartate transaminase (AST), alanine transaminase (ALT) and bilirubin in plasma were measured by standard laboratory techniques.

Isolation of liver plasma membranes and determination of enzyme activities. Plasma membranes were isolated by a procedure adapted from the one described by Emmelot et al. (19). Five grams of liver tissue, pooled from three mice, was cut in small pieces in 25 ml 1mM $NaHCO_3$, pH 7.4 with 17 mg/l PMSF (phenylmethylsulfonylfluoride) and homogenized by seven strokes in a loose dounce (Braun, Melsungen, Germany). This homogenate was filtered through cheesecloth and diluted to a total volume of 35 ml $NaHCO_3$ per 5 grams of liver. The homogenate was then centrifuged for 10 min at 1500g. The supernatant was removed and the pellet was resuspended in 35 ml fresh $NaHCO_3$ buffer. This was centrifuged again for 10 min at 1000g. The supernatant was removed again and the remaining pellet was resuspended and centrifuged again at 1000g, for 10 min. This was repeated three times in total. The remaining pellet was resuspended in $NaHCO_3$ buffer to a total of 3.6 ml and 10 ml of 62.2% (w/w) sucrose was added under mild stirring. This suspension was divided over two ultracentrifuge tubes, and was overlayered with respectively 6 ml 44.68%, 8 ml 40.81% and 6 ml 37.02% sucrose. The tubes were filled up with 0.25 M sucrose. These gradients were centrifuged for 1.5 hr at $90.000g_{av}$ in a Centrikon TI28.38 rotor, in a Centrikon T-1080 ultracentrifuge (Kontron Instruments, Milan, Italy). The bands enriched in plasma membranes floating on the 40.8% and 44.68% layers were recovered, pooled, 4 times diluted with $NaHCO_3$ and subsequently centrifuged for 15 min at 7500g. The pellet was resuspended in 40 ml $NaHCO_3$ buffer and centrifuged for 10 min at 2500 g. The final membrane pellet was resuspended in buffer containing 300 mM sucrose, 0.2 mM $CaCl_2$, 10 mM $MgSO_4$, 10 mM Hepes pH 7.5, homogenized by 50 strokes through a syringe needle and stored immediately in liquid nitrogen.

Protein concentrations were determined according to Lowry (20). Relative enrichments of Na^+ / K^+-ATPase as a marker enzyme for the basolateral membrane fraction, alkaline phosphatase as a marker enzyme for the canalicular plasma membrane fraction and succinate cytochrome C reductase as marker for the contamination with mitochondria, i.e. the activity of these enzymes in the isolated plasma membrane preparation divided by their activity in the homogenate, was used to determine the degree of purification of the isolated membranes in the different experimental groups. Na^+/ K^+ ATPase (21), alkaline phosphatase (22) and cytochrome C reductase (23) were measured using a Uvikon 931 spectrophotometer (Kontron Instruments, Milan, Italy).

Western Blotting. Plasma membranes equivalent to 20 μg of protein were electrophoresed through a 10% polyacrylamide gel at 100 V. The proteins were electrophoretically transferred onto a nitrocellulose filter (Amersham, Little Chalfont, UK) by tank blotting. Ponceau S staining was performed to check equal protein transfer. The filters were blocked overnight at 4°C in a solution of Tris-buffered saline with 0.1% Tween and 4% skim-milk powder pH 7.4. The blots were incubated with the primary antibody Ntcp (K4) in a 1:10.000 dilution for 3 hrs at room temperature, washed and immune complexes were detected using horseradish peroxidase-conjugated donkey anti rabbit IgG by the ECL Western blotting kit (Amersham, Little Chalfont, UK). Protein density was determined by scanning the blots using an Image Master VDS system (Pharmacia Biotech, Upsalla, Sweden)

Transport studies. Transport studies were carried out in plasma membrane vesicles using a rapid filtration technique (24). Five μl membrane vesicles (15 μg protein) were preincubated at 25°C for 1 min. Uptake was initiated by addition of 20 μl prewarmed incubation medium (final concentration: 100 mM NaCl or KCl, 100mM sucrose, 10 mM Hepes pH 7.5, 0.2 mM $CaCl_2$ and 10 mM $MgSO_4$, bovine serum albumin (BSA) 1 mg/ml , [^{3}H]-taurocholate was added in different concentrations) to the membranes. Uptake was performed at 25°C. Uptake was stopped by adding 750 μl of ice-cold stop solution (100 mM sucrose, 100 mM KCl, 10 mM Hepes pH 7.5, 0.2 mM $CaCl_2$ and 10 mM $MgSO_4$) to the incubation medium. The sample was immediately filtered through a 0.45 μm Millipore filter (Millipore, Bedford, MA) that was prewashed with 1 ml stop solution containing 1 mM unlabeled taurocholate, and subsequently washed twice with 4 ml ice-cold stop solution. The filters were dissolved in Ultima gold MV scintillation fluid (Packard Instruments, Dowers Grove, IL) and counted in a liquid scintillation counter type Packard 1500 (Packard Instruments, Dowers Grove, IL).

Northern Blotting. Total RNA was isolated according to Chromczynski and Sacchi (25), separated on agarose formaldehyde gel and transferred to a nylon membrane, Hybond N (Amersham, Little Chalfont, UK), by overnight blotting. cDNA probes were labelled using a random primed labelling kit to a specific activity of 10^8-10^9 cpm/μg. Blots were prehybridized in hybridization solution (0.5 M $NaHPO_4$, 1 mM Na_2EDTA and 7 % SDS, pH 7.2) and 100 μg herring sperm DNA per ml, and hybridized at 65°C overnight at 1-2 *10^6 cpm/ml in hybridization solution. They were washed twice for 15 min in

2* SSC washing buffer (0.3 M NaCl, 30 mM Na-citrate and 1% SDS, pH 7.0) at 65°C and subsequently two times in 1* SSC washing buffer (0.15 M NaCl, 15 mM Na-citrate and 1% SDS ,pH 7.0) at 65°C. Activities were corrected for concentration differences, using 28S rRNA as an internal control

Reverse transcription. Total RNA was isolated using the Rneasy kit (QIAGEN AG, Basel, Switserland) and 1 μg samples were reverse transcribed with oligo (dT) primers and 15 units of AMV reverse transcriptase (promega, Madison WI) in a 20 μl reaction volume.

Competative PCR. Primers specific for the mouse Ntcp (5'-GGTTCTCATTCCTTGCGCCA-3', bp 535-554; 5'-GCATCTTCTGTTGCAGCAGC-3', bp 1026-1007) were linked to a 600 bp sequence derived from the neomycin gene by PCR using the following primers;
5'-GGTTCTCATTCCTTGCGCCACCCTGAATGAACTGCAGGAC-3' forward
5'-GCATCTTC TGTTGCAGCAGCAGGCGATGCGCTGCGAATCG-3' reverse.
This PCR product was re-amplified with the Ntcp specific primers resulting in a 640 bp fragment which was purified and used as a heterologous competitor fragment for Ntcp. Coamplification of liver cDNA and competitor fragment yielded two PCR products of 491 bp (Ntcp) and 640 bp (competitor). Samples of the reverse transcribed reaction corresponding to 10 ng of total RNA were amplified along with competitor cDNA corresponding to 2, 1, 0.5, 0.25, 0.125 or 0.0625 amol in a 50 µl PCR reaction that contained 0.2 mM dNTPs, 0.4 M of each Ntcp specific primer, 10 mM KCl, 10 mM$(NH_4)_2SO_4$, 2 M $MgSO_4$, 20 mM Tris-HCl (pH8.75), 0.1% Triton X-100, 0.1 mg/ml BSA and 2.5 U TaqPlus Long polymerase mixture (Stratagene GmbH, Heidelberg, Germany). Cycle conditions were: 2 minutes denaturation at 95°C, 30 cycles of 45 seconds at 95 °C, 45 seconds annealing at 50°C, 1 minute elongation at 72°C, and final elongation for 5 minutes at 72 °C. Ten microliters of the reaction were separated on a 1% TAE agarose gel and after ethidium bromide staining competitor and Ntcp specific bands of equal intensities were determined visually.

Statistics. Data are expressed as mean ± SD for the indicated number of experiments. Statistical analysis between the experimental groups was assessed using Student's two tailed *t* - test. Statistical significance was considered at p-values of < 0.05.

RESULTS

Serum parameters. The plasma levels of bile salts, bilirubin and the liver-function markers AST and ALT are summarized in Table 1. Plasma concentrations of bile salts are elevated significantly, i.e. by 100% in (-/-) mice compared with (+/+) controls. As shown earlier (9,13), AST en ALT activities are markedly higher in the knockout mice than in controls. A smaller, but significant increase was found for serum bilirubin.

	AST (IU/L)	*ALT (IU/L)*	*Bilirubin (μM)*	*Bile Salts (μM)*
mdr2 (+/+)	78 ± 24	29 ± 6	3.7 ± 0.5	21 ± 4.2
mdr2 (-/-)	203 ± 64 [a]	217 ± 68 [a]	5.7 ± 0.7[a]	45 ± 25.6[a]

Data are given as means ± SD, n=9 in each group.
a: significantly different from control p<0.01

Table 1. Plasma parameters of liver function in male wild type and mdr2 Pgp-deficient mice.

Enzyme activities in homogenates and enrichment of marker enzymes in isolated membranes. Table 2 shows the activities of marker enzymes in the experimental groups. Absolute Na^+/K^+ ATPase activities in the homogenates are significantly lower in the (-/-) mice than in the controls, i.e. 3.12 and 1.84 μmol/ mg protein / hr respectively. The hepatic activity of alkaline phosphatase, on the other hand, is increased in the (-/-) mice. The activities of succinate cytochrome C reductase are not different in both groups of mice.

Enrichments of the membrane-bound marker enzymes relative to the liver homogenates are summarized in Table 3. Similar enrichments were found for the experimental groups and the contamination with mitochondria, as indicated by the loss of succinate cytochrome C reductase, is low in both groups.

	Na^+/K^+ATPase	*alkaline phosphatase* ($\mu mol \cdot mg^{-1} \cdot h^{-1}$)	*succinate cytochrome C reduct.*
mdr2 (+/+)	3.12 ± 0.44	0.46 ± 0.05	6.89 ± 0.48
mdr2 (-/-)	1.84 ± 0.41[a]	1.10 ± 0.21[a]	6.64 ± 0.57

Data are given as means ± SD, n=3 in each group, triplicate determinations
a: significantly different from control p< 0.05

Table 2. Activities of marker enzymes in liver homogenates of wild type and mdr2 Pgp-deficient mice.

	Na^+/K^+ATPase	*alkaline phosphatase*	*succ. cyt. C reduct.*
mdr2 (+/+)	14.8 ± 2.1	13.5 ± 1.6	0.15 ± 0.01
mdr2 (-/-)	16.5 ± 3.7	16.3 ± 3.0	0.35 ± 0.10

Data are given as means ± SD, n=3 in each group, triplicate determinations

Table 3. Enrichment of marker enzymes in plasma membranes isolated from livers of male wild type and mdr2 Pgp-deficient mice.

Transport studies. Km and Vmax values of taurocholate uptake were determined by measuring the initial uptake velocity over a concentration range from 10 to 160 μM in plasma membranes isolated from (+/+) and (-/-) mice. Taurocholate uptake is clearly decreased in (-/-) mice (Figure 1). Because uptake in the presence of a K^+ gradient is considered to represent the passive component of transport, the difference between Na^+-mediated and K^+-mediated transport, shown in Figure 1, represents the Na^+-dependent, carrier-mediated portion of transport across the membrane. Analysis of the initial uptake rates at various concentrations of taurocholate in male (+/+) and (-/-) mice revealed a marked decrease in the Vmax of taurocholate uptake, i.e. from 436 ± 143 in (+/+) to 177 ± 43 pmol/ mg protein/ 5 sec ($p<0.05$) in (-/-) mice. In contrast, the Km values (51 ± 17 and 41 ± 15) did not show a significant difference between the groups. These results indicate a decrease in the number of functional Na^+-dependent taurocholate transporters with a similar affinity for its substrate in isolated plasma membranes of mdr2 Pgp-deficient mice.

Ntcp protein. To determine if decreased Na^+-dependent transport activity in plasma membranes of mdr2 (-/-) mice is associated with decreased levels of Ntcp, we measured Ntcp protein levels in plasma membranes isolated from (+/+) and (-/-) mice. Figure 2 shows a representative Western blot showing three different membrane preparations of both groups of mice. As determined by scanning the blots, the relative Ntcp protein levels in (-/-) mice are 4-6 times lower than in (+/+) mice.

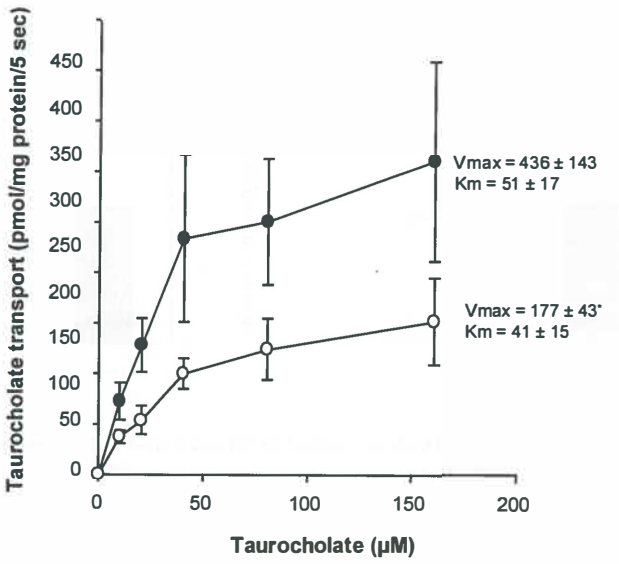

Figure 1. Kinetics of sodium-dependent taurocholate transport in plasmamembranes isolated from (+/+), closed symbols and (-/-), open symbols, mice. *Initial uptake velocity of taurocholate was measured over a range from 10 to 160 µM. Each point represents the mean of triplicate measurement in three different membrane preparations.*
** p< 0.05 compared with mdr2 (+/+).*

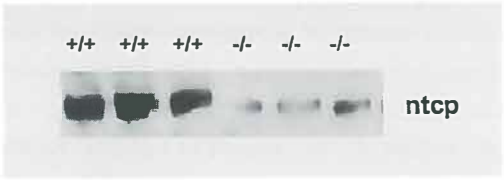

Figure 2. Ntcp protein mass in plasma membranes isolated from wildtype (+/+) and mdr2 Pgp deficient mice (-/-). *Three different membrane preparations of each group are shown*

Steady-state mRNA levels. To investigate whether changes in protein expression of Ntcp can be explained by an altered gene expression, as is the case in models of experimental cholestasis, steady state mRNA levels were compared using Northern blot analysis (Figure 3a). To exclude effects of differential RNA loading onto the gel, the 28S rRNA levels were determined for normalization. Interestingly, this ratio was about one in both groups indicating that no regulation takes place at transcriptional level. Likewise, no differences in *Ntcp* mRNA levels were detected by competitive RT-PCR analysis (figure 3b). Obviously, these results indicate that posttranscriptional down-regulation of Ntcp occurs in these knockout mice.

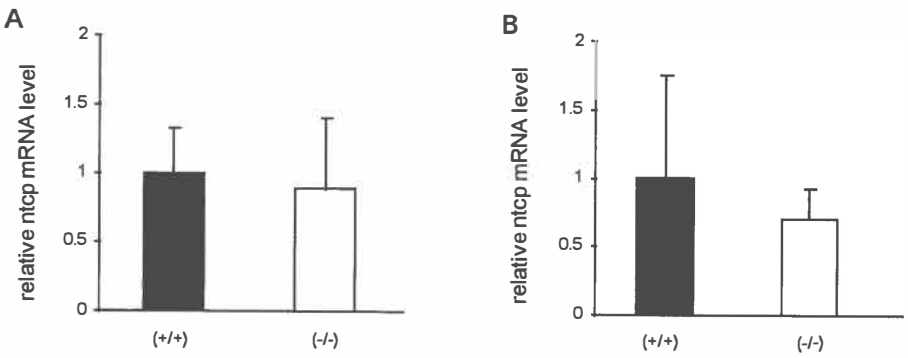

Figure 3. Relative ntcp mRNA levels in control (+/+) mice and mdr2 knockout (-/-) mice. *Ntcp mRNA levels were determined by Northern blotting (a) or competitive RT-PCR (b). Values were normalized by using a 28S rRNA probe (a) or by setting the control mRNA amount to 1 (b). Each bar represents the mean of triplicate total RNA isolations from different livers (a). Competitive RT-PCR was performed with total RNA from 5 (-/-) and 3 (+/+) mice.*

DISCUSSION

This study is the first to describe a clear down-regulation of Ntcp protein levels and activity in a non-cholestatic animal model. In recent years expression and activity of sodium-dependent bile salt transport has been studied in a number of experimental models of cholestasis (2-6). Without exception, cholestasis was found to be associated with a clear down-regulation of Ntcp, in most cases on activity, protein as well as on mRNA levels. The molecular background of these changes in Ntcp levels and activity are not yet understood. The presence of a putative BS responsive element in the promotor region of rat*Ntcp* (26,27) suggests that high intracellular bile salt concentrations as occur during cholestasis may be responsible for down-regulation. In fact, Gartung et al. (8) reported an inverse relationship between plasma bile salt levels on Ntcp protein levels in rat liver. In addition, it was shown that cytokines like TNFα and IL1β are able to down-regulate Ntcp *in vivo* and *in vitro* (3,4,28). Both bile salt induced- and cytokine induced-phenomena can play a role in the different cholestatic models. It has been proposed that down-regulation of Ntcp in conditions associated with cholestasis protects the hepatocyte from intracellular accumulation of toxic bile salts (1,8,29). The *mdr2* knock out mice represent a non-cholestatic model when judged on the basis of bile flow. However, these mice do present with a "cholestatic" plasma, with elevated transaminases and alkaline phosphatase, increased bilirubin and increased bile salt levels.

Mdr2 Pgp-deficiency in mice leads to formation of phospholipid- and cholesterol-depleted bile. Bile salt secretion is not affected in these mice. Bile flow is increased, which has been attributed to a higher choleretic potency of biliary bile salts in the absence of mixed micelle formation. This view is supported by the fact that glutathione secretion, which is considered to be responsible for the generation of BSIF in rodents, is diminished in (-/-) mice (9). The increased flow of water probably is generated at the level of the bile ducts that are strongly proliferated. The composition of the bile salt pool is not changed, but pool size appears to be increased in (-/-) mice. By infusing increasing amounts of taurocholate it has been shown that (-/-) mice transport bile salts at or near their maximal secretory capacity (S_{RM}) under normal circumstances (17). This is in marked contrast to the situation in control mice in which the S_{RM} is about 2-fold higher than the normal physiological output rate (17).

A decrease in the Vmax for taurocholate transport with unchanged Km was found in plasma membranes isolated from *mdr2* knockout mice. The kinetic values obtained in the present study are in agreement with those reported before for Ntcp-mediated transport in rat liver (24,30). However, we did find a discrepancy between transport data and Ntcp protein levels as determined by Western blotting. Whereas Ntcp protein levels were 4-6 times lower, we found the Vmax of taurocholate transport to be decreased only 2-fold. One explanation for this divergence can be the difference in sensitivity of the two methods applied. Another explanation is that there is an additional, as yet unidentified, sodium-dependent taurocholate transporter in hepatic plasma membranes of the mouse. In fact, an alternative splice variant of Ntcp, called Ntcp-2, has been found in mouse liver (31). However, Ntcp-2 mRNA is about 25 times less abundant than that of Ntcp-1, both in (+/+) and (-/-) mice and therefore is probably of no physiological importance (data not shown). Alternatively, since we have used total plasma membrane preparations in our study, it may be that bile salt transporters present in bile ductuli contribute to overall Na^+-dependent transport in these studies. Recently, it was shown that the intestinal sodium-dependent bile salt transporter (ibst) is also expressed in apical membrane of the bile duct epithelium in rats (32,33). It was suggested (33) that uptake of bile salts by the bile duct cells may contribute to a cholehepatic shunt, in which bile salts taken up by cholangiocytes are returned to the hepatocytes via the hepatobiliary plexus. Since bile ducts are strongly proliferated in (-/-) mice, it may be that ibst is highly expressed in these animals. If ibst is indeed upregulated in (-/-) mice, this could partly explain the observed discrepancy between transport activity and Ntcp levels. At the moment, nothing is known about the kinetics of the ileal bile salt transporter in mice. The reported Km value of the intestinal rat bile salt transporter (33μM) (34) and the bile duct ibst (43 μM) (35) are in the same order of magnitude, as that of Ntcp (25-46μM) (15,30) in rats. Whether this is also the case for mice remains to be determined.

The decrease in Ntcp protein levels was, to our surprise, not accompanied by a similar decrease in mRNA levels, in contrast to the situation in the cholestatic models mentioned previously (2-6). This suggests posttranscriptional mechanisms to be involved in Ntcp down-regulation in this particular

model. In fact, a discrepancy between the reduction in mRNA levels and protein levels was also noted by Gartung *et al.*(5) in bile duct ligated rats, although in this case mRNA levels are clearly reduced. Together, these findings suggest that regulation of Ntcp may have both transcriptional and posttranscriptional components and that their contribution may depend on the severity of cholestasis. It is tempting to speculate that the situation in mdr2 Pgp-deficient mice represents a pre-cholestatic condition. As mentioned previously, biliary bile salt secretion appears to proceed at, or very near, its maximal capacity. In fact, we have recently shown that feeding of cholesterol associated with a compensatory increase in bile salt synthesis rapidly leads to elevations in plasma bile salt concentrations and to hyperbilirubinemia in (-/-) mice (36). This observation supports the view that these mice may be "on the edge" of developing cholestasis when the system is stressed. Contributing to, or even underlying this situation may be a constant exposure of the (-/-) livers to small amounts of cytokines, due to the ongoing inflammation. As also indicated before, cytokines as well as inflammation induced by endotoxin rapidly lead to down-regulation of Ntcp as well as canalicular transporters involved in bile formation (3,4,28).

Thus, in the scenario outlined above, it may be that posttranscriptional down-regulation of Ntcp may be an initial event in cells that are almost cholestatic, perhaps aimed to protect the cells from high intracellular bile salt loads. In the case of a exacerbation, e.g. due to obstruction of the bile ducts, transcriptional down-regulation also jumps in to fully down-regulate Ntcp. The questions that remain to be answered concern the signals for both ways of regulation. Whereas there are strong indications that intracellular bile salt concentrations affect *Ntcp* gene transcription (5,8), mechanisms responsible for posttranscriptional regulation remain fully speculative at this moment.

A factor that could contribute is the Na^+/K^+ATPase activity in the sinusoidal membrane. We found in (-/-) mice a two-fold decrease in Na^+/K^+ATPase activity. Simultaneously decreased activities of Na^+/K^+ATPase and Ntcp are also found in cholestasis caused by ethinylestradiol in rats (6,37). Of course the question still remains whether decreased Na^+/K^+ATPase activity affects Ntcp levels, or *vice versa*.

In conclusion we demonstrate posttranscriptional down-regulation of Ntcp protein levels and transport activity in male mdr2 Pgp-deficient mice. We propose that this mechanism of down-regulation of Ntcp plays a role in the hepatic protection against high intracellular bile salt loads in the "near cholestatic" condition that has to be handled by these mice.

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REFERENCES

1.Koopen NR, Wolters H, Müller M, Schippers IJ, Havinga R, Roelofsen H, Vonk RJ, Stieger B, Meier PJ, Kuipers F. Hepatic bile salt flux does not modulate level and activity of the sinusoidal Na^+-taurocholate cotransporter (ntcp) in rats. J Hepatology 1997;27:699-706.
2.Bolder U, Ton-Nu HT, Schteingart CD, Frick E, Hofmann AF. Hepatocyte transport of bile acids and organic anions in endotoxemic rats: impaired uptake and secretion. Gastroenterology 1997;112:214-24.
3.Green RM, Beier DR, Gollan JL. Regulation of hepatocyte bile salt transporters by endotoxin and inflamatory cytokines in rodents. Gastroenterology 1996;111:193-8.
4.Moseley RH, Wang W, Takeda H, Lown K, Shick L, Ananthanarayanan M, Suchy FJ. Effect of endotoxin on bile transport in rat liver: a potential model for sepsis-associated cholestasis. Am J Physiol 1996;271:G137-46.
5.Gartung C, Ananthanarayanan M, Rahman M, Schuele S, Nundy S, Soroka CL, Stolz A, Suchy FJ, Boyer JL. Down-regulation of expression and function of the rat liver Na^+/Bile acid cotransporter in extrahepatic cholestasis. Gastroenterology 1996;110:199-209.
6.Simon FR, Fortune J, Iwahashi M, Gartung C, Wolkoff AW, Sutherland E. Ethinyl estradiol cholestasis involves alterations in expression of liver sinusoidal transporters. Am J Physiol 1996;271:G1043-52.
7.Green RM, Gollan JL, Hagenbuch B, Meier PJ, Beier DR. Regulation of hepatocyte bile salt transporters during hepatic regeneration. Am J Physiol 1997;273:G261-7.
8.Gartung C, Schuele S, Schlosser SF, Boyer JL. Expression of the rat liver Na^+/taurocholate cotransporter is regulated by in vivo retention of biliary constituents but not their depletion. Hepatology 1997;25:284-90.
9.Smit JJM, Schinkel AH, Oude Elferink RPJ, Groen AK, Wagenaar E, van Deemter L, Mol CAAM, Ottenhoff R, van der Lugt NMT, van Roon MA, van der Valk MA, Offerhaus GJA, Berns AJM, Borst P. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell 1993;75:451-62.
10.Reichen J, Simon FR. ; Arias I, Boyer J, Fausto N, Jakoby W, Schachter D, Shafritz D, editors.The liver: Biolology and Pathobiology. 3rd ed. New York: Raven Press,Ltd. 1994; 68, Cholestasis. p. 1291-326.
11.Fallon MB, Anderson JM, Boyer JL. ; Schiff I, Schiff E, Lippinger J, editors.Diseases of the Liver. 7th ed. Philadelphia: J.B. Lippincott Company, 1993; 13, Intrahepatic cholestasis. p. 343-61.
12.Oelberg D, Lester R. Cellular mechanisms of cholestasis. Ann Rev Med 1986;37:279-317.
13.van Nieuwkerk CMJ, Groen AK, Ottenhoff R, van Wijland MJ, van den Berg Weerman MA, Tytgat GN, Offerhaus GJA, Oude Elferink RPJ. The role of bile salt composition in liver pathology of mdr2 (-/-) mice: differences between males and females. J Hepatology 1997;26:138-45.
14.Benedetti A, Alvaro D, Bassoti C, Gigliozzi A, Ferretri G, La Roza T, Di Saro A, Baiocchi L, Jezequel AM. Cytotoxicity of bile salts against biliary epithelium: a study in isolated bile ductule fragments and isolated perfused rat liver. Hepatology 1997;26:9-21.
15.Hagenbuch B, Stieger B, Foguet M, Lübbert H, Meier PJ. Functional expression cloning and characterization of the hepatocyte Na+/ bile acid cotransport system. Proc Natl Acad Sci 1991;88:10629-33.
16.Stieger B, Hagenbuch B, Landmann L, Höchli M, Schroeder A, Meier PJ. In situ localization of the hepatocytic Na+/ Taurocholate cotransporting polypeptide in rat liver. Gastroenterology 1994;107:1781-7.
17.Oude Elferink RPJ, Ottenhoff R, van Wijland MJ, Smit JJM, Schinkel AH, Groen AK. Regulation of biliary lipid secretion by mdr2 P-glycoprotein in the mouse. J Clin Invest 1995;95:31-8.
18.Murphy GM, Billing BH, Baron DN. A fluorimetric and enzymatic method for the estimation of serum total bile acids. J Clin Pathol 1970;23:594-8.
19.Emmelot P, Bos CJ, van Hoeven RP, van Blitterswijk WJ. ; Fleisher S, Packer L, editors.Methods in enzymology XXXI. New York, San Fransisco, London: Academic Press, 1974;Isolation of plasmamembranes from rat and mouse livers and hepatomas. p. 75-90.
20.Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.

21.Scharschmidt BF, Keeffe EB, Blankenship NM, Ockner RK. Validation of a recording spectrophotometric method for measurement of membrane-associated Mg- and NaK-ATPase activity. J Lab Clin Med 1979;93:790-9.
22.Farrell GC, Duddy SK, Kass GEN, Llopis J, Orrenius S. Release of Ca^{2+} from the endoplasmic reticulum is not the mechanism for bile acid induced cholestasis and hepatotoxicity in the intact rat liver. J Clin Invest 1990;85:1255-9.
23.Goldberg JA, Rutenberg AM. The colorimetric determination of leucine aminopeptidase in urine and serum of normal subjects and patients with cancer and other diseases. Cancer Res 1958;11:283-91.
24.Meier PJ, Meier-Abt AS, Barret C, Boyer JL. Mechanisms of taurocholate transport in canalicular and basolateral rat liver plasma membranes. J Biol Chem 1984;259:10614-22.
25.Chromczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Ann Biochem 1987;162:156-8.
26.Karpen SJ, Sun AQ, Kudish B, Hagenbuch B, Meier PJ, Ananthanarayanan M, Suchy FJ. Multiple factors regulate the rat liver basolateral sodium dependent bile acid cotransporter gene promoter. J Biol Chem 1996;271:15211-21.
27.Karpen SJ, Ananthanarayanan M, Potselueva T, Suchy FJ. Bile acids suppress the rat liver Na^+-dependent bile acid cotransporter gene promoter. [Abstract] Hepatology 1995;22:(4)318a
28.Whiting JF, Green RM, Rosenbluth AB, Gollan JL. Tumor necrosis factor -alfa decreases hepatocyte bile salt uptake and mediates endotoxin-induced cholestasis. Hepatology 1995;22:1273-8.
29.Setchell KDR, Rodrigues CMP, Clerici C, Solinas A, Morelli A, Gartung C, Boyer JL. Bile acid concentrations in human and rat liver tissue and hepatocyte nuclei. Gastroenterology 1997;112:226-35.
30.Zimmerli B, Valantinas J, Meier PJ. Multispecificity of Na+-dependent taurocholate uptake in basolateral (sinusoidal) rat liver plasma membrane vesicles. J.Pharmacol.Exp.Therap. 1989;250:301-8.
31.Hagenbuch B. Alternative splicing results in two transcripts for the mouse liver Na^+/taurocholate cotransporting polypeptide. [Abstract] Hepatology 1997;26:(4)295A
32.Lazaridis KN, Pham L, Tietz P, Marinelli RA, de Groen P, Levine S, Dawson PA, LaRusso F. Rat cholangiocytes absorb bile acids at their apical domain via the ileal sodium dependent bile acid transporter. J Clin Invest 1997;100:2714-21.
33.Gurantz D, Hofmann AF. Influence of bile acid structure on bile flow and biliary lipid secretion in the hamster. Am J Physiol 1984;247:G736-48.
34.Wong MH, Oelkers P, Craddock AL, Dawson PA. Expression cloning and characterization of the hamster ileal sodium dependent bile acid transporter. J Biol Chem 1994;269:1340-7.
35.Alpini G, Glaser S, Rodgers R, E.D., Phinizy JL, Robertson W, Lasater J, Caligiuri A, Tretjak Z, Lesage GD. Functional expression of the apical Na^+-dependent bile acid transporter in large but not small rat cholangiocytes. Gastroenterology 1997;113:1734-40.
36.Voshol PJ, Koopen NR, Havinga R, Oude Elferink RPJ, Groen AK, Kuipers F. Hepatic actions of dietary cholesterol in mdr2 P-glycoprotein-deficient mice. [Abstract] Hepatology 1997;26:(4)294A
37.Berr F, Simon FR, Reichen J. Ethinylestradiol impairs bile salt uptake and Na^+-K^+pump function of rat hepatocytes. Am J Physiol 1984;274:G437-43.

Chapter 6

Toxic effects of dietary cholesterol in mdr2 p-glycoprotein-deficient mice lacking biliary lipids

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Submitted

ABSTRACT

Mdr2 P-glycoprotein (Pgp) deficiency in mice leads to formation of phospholipid- and cholesterol-depleted bile, while bile salt secretion is unaffected. Plasma HDL cholesterol levels in chow-fed *mdr2* Pgp-deficient (-/-) mice are only 45% of control values. To investigate whether this phenotype is caused by insufficient delivery of phospholipids and/or cholesterol into the intestine, male and female (-/-) and wildtype (+/+) mice were fed isocaloric semi-synthetic diets with or without additional egg phosphatidylcholine for two weeks. Subsequently, cholesterol was added to the diets for another week. Addition of phospholipids and/or cholesterol did not normalize plasma lipoprotein levels in (-/-) mice. These data indicate that low plasma lipid levels in (-/-) mice are not directly related to the absence of lipids in the intestine and indicate a specific role for biliary lipids in the regulation of plasma HDL levels. Surprisingly, dietary cholesterol led to a marked hyperbilirubinemia in male (+100%) and female (+500%) (-/-) mice, both in the presence and absence of phospholipid. Even more striking was the increase in plasma bile salt levels after cholesterol feeding, i.e., by 200% in males and by 1250% in females. These effects were not observed in (+/+) mice of either sex. Bile flow, biliary bile salt secretion nor biliary bilirubin secretion were affected in (-/-) mice by cholesterol feeding, indicating that no overt cholestasis was induced. Increased plasma bile salt levels may be related to reduced levels of Na+-taurocholate cotransporting protein (ntcp) in liver plasma membranes of (-/-) mice. This report is the first to describe induction of hypercholanemia and hyperbilirubinemia by dietary cholesterol *in vivo*, delineating the important role of biliary lipid secretion in normal hepatic functioning.

INTRODUCTION

The biliary pathway is considered the major route for removal of excess cholesterol from the body (1,2). Cholesterol, either as such or after its conversion to bile salts, is expelled through bile into the intestinal lumen followed by fecal elimination. However, the majority of bile salts and a considerable fraction of biliary cholesterol, approximately 65% (3), is reabsorbed from the intestine. Bile salts are transported back to the liver via the portal system for resecretion into bile. The physiological importance of this so-called enterohepatic circulation of bile salts in the regulation of hepatic bile salt and cholesterol synthesis (1,4), LDL receptor activity (5) and VLDL formation (6,7) is well-established. Less is known about the physiological significance of biliary cholesterol reabsorption. In this respect, it is of importance to note that the amount of cholesterol entering the intestine via bile, up to 1200 mg/day in humans (1), is typically two to three times larger than the amount of dietary cholesterol. Biliary cholesterol is accompanied by a six- to eight-fold excess of bile specific phospholipids, mainly phosphatidylcholine with 16:0-18:1, 16:-18:2 or 16:0-20:4 configuration. These phospholipids are essential for secretion of biliary cholesterol (8,9) and for protection of the biliary tree against the cytotoxic actions of bile salts (10-12). In addition, it has been described that biliary phospholipids have a stimulatory action on formation of chylomicrons by enterocytes and, therefore, may be essential for efficient absorption of dietary fat (13,14). Little is known about the role of biliary cholesterol and phospholipids *per se* in the maintenance of cholesterol homeostasis and in the regulation of plasma cholesterol levels. This is mainly due to the fact that secretion of these lipids is coupled to that of bile salts. As a consequence, it has not been possible to study the actions of biliary lipids independent from those of bile salts in the *in vivo* situation. Recently generated *mdr2* P-glycoprotein (Pgp)-deficient mice (8-10) provided us with the tool to address this issue. *Mdr2* Pgp acts as an ATP-dependent "flippase" translocating phosphatidylcholine across the bile canalicular membrane (8,10). *Mdr2* P-gp-deficient mice are unable to secrete phospholipids into bile and, as a consequence, do not secrete cholesterol under physiological conditions (8,10). Bile salt secretion, on the other hand, is not affected in these animals (8,10).

In a previous study (15), we observed that mice homozygous for *mdr2* gene disruption (-/-) have strongly reduced plasma HDL-cholesterol levels (- 65%) when fed normal laboratory. Heterozygous (+/-) mice show a 25% reduction in plasma HDL-cholesterol compared with wild type controls (15). Surprisingly, apolipoprotein B48 and B100 levels are increased in (-/-) mice (15). We hypothesized that reduced HDL cholesterol levels in (-/-) mice are due to the absence of biliary lipids in the intestine leading to impaired chylomicron formation. Our aim for the present study was to evaluate whether or not the phenotype of *mdr2* Pgp-deficient mice with respect to plasma lipoprotein levels could be restored by addition of phosphatidylcholine and/or cholesterol to the diet. For this purpose we supplied mice with dietary egg-phosphatidylcholine (PC), the composition of which resembles that of biliary PC, with or without additional cholesterol. PC and cholesterol were added to the diets in

amounts equivalent to respectively ten-times and thirty-times the daily biliary load of these lipids in control mice. Since supplementation of PC to the diet has been shown to improve liver function in animal models of alcohol-induced hepatotoxicity (16,17) and because (-/-) mice develop liver pathology due to the formation of lipid-free bile (11,12), we also evaluated parameters of liver function in these experiments. No effects of dietary PC and cholesterol on plasma lipid levels in (-/-) mice were found. However, cholesterol feeding resulted in a marked hyperbilirubinemia and hypercholanemia in (-/-) mice, with elevated plasma alkaline phosphatase. To the best of our knowledge, this is the first study to describe induction of hypercholanemia and hyperbilirubinemia by dietary cholesterol in an *in vivo* model.

MATERIALS AND METHODS

Animals: Control (+/+) and *mdr2* knockout (-/-) mice, 32 males and 32 females, were obtained from the breeding colony at the Academic Medical Center, Amsterdam. Animals were kept on a semi-purified diet, the composition of which is shown in table 1.

Compound	*% (w/w)*
Cerelose	54.3
Casein	20.0
Corn starch	10.0
Cellulose	5.0
Soya-oil	5.0
Choline	0.4
Vitamin/minerals etc.	5.3

Table 1. Composition of the semi-purified diet (reference diet) as purchased from Hope Farms BV, Woerden, The Netherlands.

(Hope Farms BV, Woerden, The Netherlands), for two weeks before starting the experiment. After these two weeks, the mice were randomly divided into two groups, one group (16 mice of either sex) continuing on the same semi-purified diet and the other group receiving the same diet supplemented with 11.3 mmol/kg PC. Animals received these diets for a period of two weeks and were than subdivided again, with half of the animals remaining on their diet and the other half receiving the same diet supplemented with 0.25% cholesterol (6.5 mmol/kg), resulting in n = 8 per dietary group for both sexes (see Figure 1). Food and water were available *ad libitum*. The experimental procedure was approved by the Ethical Committee for Animal Experiments of the University of Groningen.

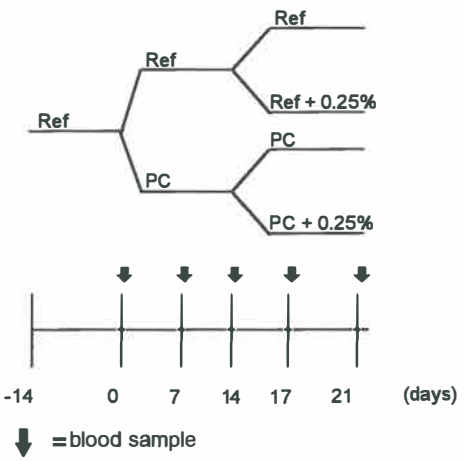

Figure 1: Protocol design of the experiment, showing the different dietary groups and the time points of blood sampling during the experiment.

Experimental procedures. Blood samples were collected at baseline (day 0), after one (day 7) and two (day 14) weeks on semi-purified diets with or without PC and after 3 (day 17) and 7 (day 21) days of cholesterol feeding, as indicated in Figure 1. Small blood samples at days 0, 7, 14 and 17 were collected by tail bleeding under light halothane anesthesia. At day 21, the gallbladder of mice was canulated under Hypnorm (fentanyl/fluanisone, 1 mL/kg) and Diazepam (10 mg/kg) anesthesia and bile was collected during a 30 minute period. Bile production was assessed by weight. Subsequently, a large blood sample was collected by cardiac puncture.

Three mice of each group were used for investigating liver pathology and for RNA isolation. Mice were killed by cardiac puncture and the liver was perfused with ice-cold saline, excised, weighed and used for examining liver morphology after paraformaldehyde fixation and HE staining, or, after snap freezing in liquid isopentane, for membrane isolation and RNA isolation.

Western blot analysis. Part of the collected livers was used to isolate a total membrane fraction as previously described (18), for assessment of Na+-taurocholate cotransporting protein (ntcp) levels. Protein (75 μg) was separated using SDS gel electrophoresis (13% Ready gels, BioRad Laboratories, Hercules, CA, USA) and subsequently blotted onto nitrocellulose (Hybond-ECL, Amersham, UK) and probed with anti-ntcp-immunoglobulin IgG K4 (kindly provided by B. Stieger, Zürich, Switzerland (19)). Immune complexes were detected using horseradish peroxidase-conjugated donkey anti-rabbit-IgG by ECL Western blotting Kit (Amersham, UK).

Northern blot analysis. RNA was isolated for analysis of steady state levels of HMG-CoA synthase, cholesterol 7α-hydroxylase and cholesterol 27-hydroxylase by Northern blotting (20). Levels of the different mRNA's where expressed in arbitrary units relative to ribosomal 18S mRNA and presented as percentage of control values. Blot density was determined by scanning the blots using an ImageMaster VDS system (Pharmacia, Upsalla, Sweden).

Analyses. Plasma total cholesterol, free cholesterol and triglycerides (glycerol blanking) were measured using commercially available kits, according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Plasma free fatty acids and phospholipids were determined using commercial kits from WAKO (WAKO Chemicals GmbH, Neuss, Germany). Contents of cholesterol in bile and cholesterol (ester) and triglycerides in liver tissue was determined after lipid extraction (21) as described previously (22). Protein content of the liver homogenates was determined according to Lowry et al. (23), with BSA as standard. Total bile salt concentration in bile and plasma were determined by an enzymatic fluorimetric assay (24). Plasma and biliary bilirubin (total and conjugated), alanine-aminotransferase (ALAT), aspartate-aminotransferase (ASAT) and alkaline phosphatase (AP), were determined by standard procedures at the Central Clinical Laboratory at the Academic Hospital Groningen.

Liver histology. HE-stained material of the different groups were scored blindly for the following histopathological parameters in four grades of severity (0-3): mitotic activity and councilman bodies (hepatocyte damage) and portal inflammation, ductular proliferation and fibrosis (damage in the portal tract). A maximal pathology score of 15 points could be reached. This scoring system is the same as described previously (11, 12). Mean values were determined for $n = 3$ per group.

Statistical analysis. All results are presented as means ± standard deviations for the number of nimals indicated. Differences between dietary groups was determined by one-way ANOVA analysis (25), with posthoc comparison by Newmann Keuls t-test (25), for both (-/-) and (+/+) mice. Differences between (-/-) and (+/+) mice were determined by Mann Whitney U-test analysis (25) for the different dietary groups. Level of significance for all statistical analysis was set at $p < 0.05$. Analysis was performed using SPSS for Windows software (SPSS, Chicago, IL, USA)

RESULTS

Plasma lipid levels. Figure 2 shows the plasma cholesterol levels during the course of the experiment in male (+/+) and (-/-) mice on reference (panel A) or PC-supplemented (panel B) diets with and without cholesterol. PC feeding alone did not affect plasma cholesterol levels in either group. Addition of cholesterol to the reference diet led to an increase in plasma cholesterol in (+/+) mice:

this effect was diminished after 7 days of cholesterol feeding (panel A). Feeding PC and cholesterol led to increased plasma cholesterol levels that sustained after seven days in (+/+) mice (panel B). There were no changes in plasma cholesterol levels in male (-/-) on the different diets, except for a very modest increase in PC + cholesterol-fed animals.

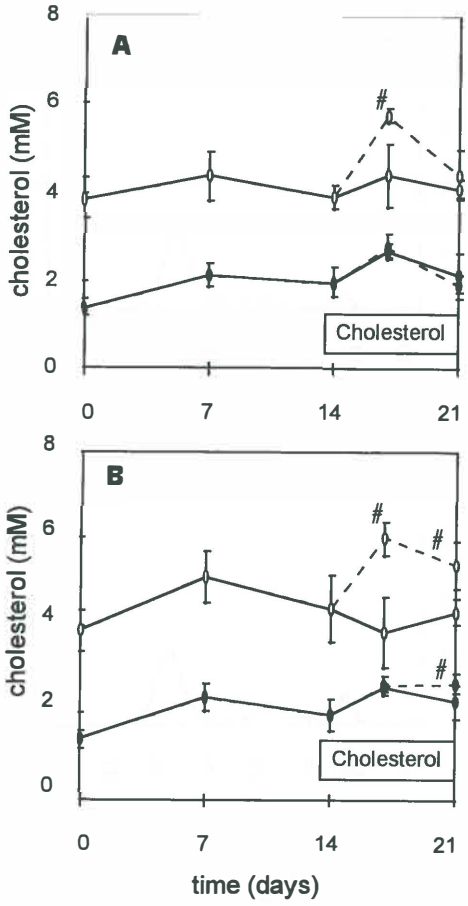

Figure 2. Plasma cholesterol levels during the experiment in male mdr2 mice on reference (panel A) and phospholipid-containing (panel B) diets. *Samples are taken on day 0, 7, 14, 17 and 21 by tail bleeding as described in the method section. Data represent mean plasma cholesterol (mM) ± SD, n = 8. Differences were analyzed by one-way ANOVA analysis with Newmann-Keuls t-test postboc analysis, (#) p < 0.05.* ❍ *mdr2 (+/+) mice,* ● *mdr2 (-/-) mice,* ——— *without cholesterol added and* ------- *with 0.25% cholesterol added*

Table 2 summarizes plasma cholesterol, cholesterol ester, triglyceride, phospholipid and free fatty acid levels in male and female (+/+) and (-/-) mice at the end of the experiment. Cholesterol and cholesterol esters were significantly lower in (-/-) mice compared with controls, in males (see also figure 2) as well as in females, in all dietary groups. There were no effects on plasma triglycerides or

free fatty acids in male or female (+/+) and (-/-) mice on either diet. Plasma phospholipids only showed an increase in male (+/+) mice fed the PC diet. Male and female (+/+) mice fed the PC + cholesterol diet had higher plasma phospholipid levels than (-/-) mice.

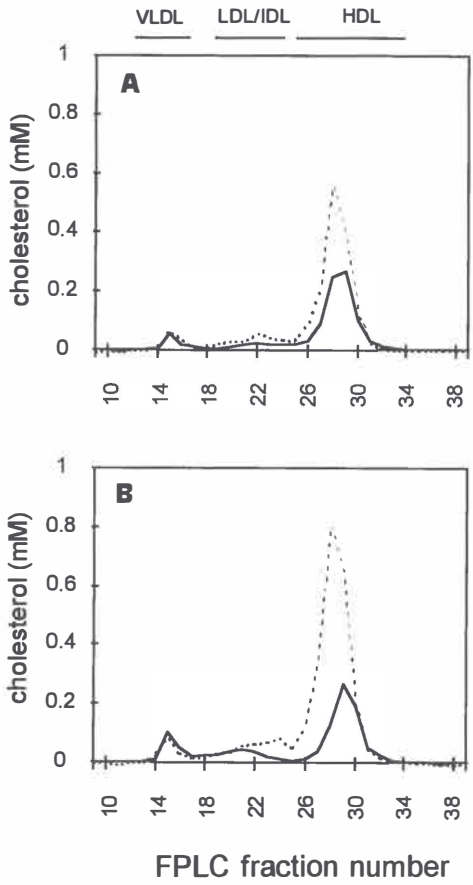

Figure 3. Fast Protein Liquid Chromatography (FPLC) separation of lipoproteins in 200 µL pooled plasma on a Superose 6B column (Pharmacia). *Panel A: (+/+) and (-/-) mice on phospholipid diet and panel B: (+/+) and (-/-) mice on phospholipid + 0.25% cholesterol diet. VLDL, IDL/LDL and HDL are indicated. ------- mdr2 (+/+) mice and —— mdr2 (-/-) mice.*

We also analyzed whether feeding of PC and/or cholesterol altered plasma lipoprotein profiles. Feeding of PC alone did not results in changes in plasma lipoprotein profiles (data not shown). Figure 3 shows the cholesterol content of plasma lipoproteins after FPLC separation for male (+/+) and (-/-) mice on the PC diet without (panel A) or with cholesterol (panel B). Feeding PC + cholesterol in (+/+) mice led to an increase in HDL cholesterol levels. Similar observations were made in female (+/+) mice. However, no changes in lipoprotein cholesterol content were induced in either male (compare Figure 3A and 3B) or female (-/-) mice by cholesterol feeding.

Hepatic cholesterol content. The absence of an effect of cholesterol feeding on plasma cholesterol in (-/-) mice could be due to absent or reduced uptake of dietary cholesterol from the intestine. We therefore measured hepatic cholesterol content as a reflection of intestinal uptake. Figure 4 shows the hepatic total cholesterol content in male and female (+/+) and (-/-) mice on the four different diets. Both (+/+) and (-/-) mice showed an increase in hepatic total cholesterol content after one week of cholesterol supplementation, either with or without PC. This increase was due to an increase in hepatic cholesterol ester content: hepatic free cholesterol remained unchanged in all groups at approximately 40-50 nmol/mg protein. Hepatic triglyceride levels increased 1.5-2 times on cholesterol-containing diets in (+/+) mice. No changes were observed in the (-/-) mice. Hepatic phospholipid levels did not differ between (+/+) and (-/-) mice on either of the four diets.

Mouse	*Diet*	*cholesterol (mM)*	*cholesterol esters (mM)*	*triglycerides (mM)*	*Phospholipids (mM)*	*Free fatty acids (mM)*
Male +/+	**Ref**	4.1 ± 0.2	3.2 ± 0.3	0.6 ± 0.2	2.0 ± 0.3	1.8 ± 0.3
	Ref + 0.25%	4.4 ± 0.2	3.2 ± 0.6	0.7 ± 0.2	2.6 ± 0.1	2.2 ± 0.2
	PC	4.3 ± 0.3	3.2 ± 0.2	0.7 ± 0.2	2.9 ± 0.4 #	1.6 ± 0.5
	PC + 0.25%	5.4 ± 0.5 #	4.3 ± 0.2 #	1.0 ± 0.5	3.0 ± 0.5 #	1.9 ± 0.3
Female +/+	**Ref**	3.3 ± 0.4	2.4 ± 0.1	0.8 ± 0.2	1.7 ± 0.1	1.8 ± 0.7
	Ref + 0.25%	3.4 ± 0.6	2.5 ± 0.3	0.4 ± 0.2 #	1.8 ± 0.1	1.5 ± 0.4
	PC	3.3 ± 0.3	2.8 ± 0.2 #	0.6 ± 0.2	2.0 ± 0.6	1.9 ± 0.7
	PC + 0.25%	3.9 ± 0.4 #	3.1 ± 0.2 #	0.8 ± 0.3	2.0 ± 0.6	1.7 ± 0.5
Male -/-	**Ref**	2.1 ± 0.5 *	1.9 ± 0.2 *	0.8 ± 0.2 *	2.1 ± 0.8	2.1 ± 0.5
	Ref + 0.25%	1.9 ± 0.2 *	1.5 ± 0.3 *	0.6 ± 0.1	1.9 ± 0.4 *	1.3 ± 0.3 * #
	PC	2.3 ± 0.4 *	2.0 ± 0.1 *	1.1 ± 0.2 * #	2.5 ± 0.4	2.0 ± 0.1

Blood samples were taken at the end of the experiment by cardiac puncture as described in the material and method section. (*) significant difference mdr2 -/- versus mdr2 +/+, Mann-Whitney U test, $p < 0.05$, $n = 8$ per group. (#) significant difference dietary group versus other dietary groups, one-way ANOVA, Newmann-Keuls t-test $p < 0.05$, $n = 8$ per group.

***Table 2. Plasma cholesterol, cholesterol ester, triglyceride, phospholipid and free fatty acid levels** in mdr2 (+/+) and (-/-) mice fed reference (Ref), reference + cholesterol (Ref + 0.25% chol), phospholipid (PC) or phospholipid + cholesterol (PC + 0.25% chol) diet.*

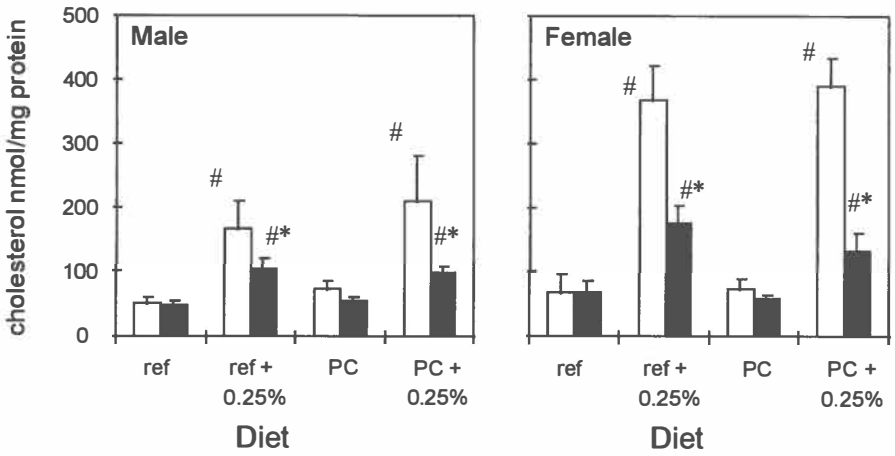

Figure 4: Hepatic cholesterol content in liver of mdr2 (+/+) and (-/-) male and female mice *on reference (ref), reference + 0.25% cholesterol (ref + 0.25%), phospholipid (PC) and phospholipid + 0.25% cholesterol (PC + 0.25%) diet. Values represent total cholesterol in nmol per mg protein, n = 5 per group. Differences between the dietary groups were determined by one-way ANOVA analysis and Newmann-Keuls t-test posthoc analysis, (#) p < 0.05.* □ *mdr2 (+/+) mice and* ■ *mdr2 (-/-) mice*

Plasma bile salt, bilirubin, ASAT, ALAT and AP levels. Cholesterol-fed (-/-) mice developed jaundice within three days. Figure 5 shows plasma bilirubin and bile salt levels of male and female (+/+) and (-/-) mice on the four experimental diets. Bile salt levels in (-/-) mice before initiation of cholesterol feeding at day 14 were significantly higher than those of sex-matched controls, as previously reported (8), whereas bilirubin levels did not differ. Feeding of PC alone did not have significant effects on plasma bile salts or bilirubin in the (-/-) mice. Feeding of cholesterol led to an strong increase in bilirubin and bile salts levels in male and female (-/-) mice, i.e., 2-8 fold for bilirubin and 5-25 fold for bile salts when compared with control-fed (-/-) mice. As shown in table 3, both the conjugated and the unconjugated fraction of plasma bilirubin significantly increased in (-/-) mice upon cholesterol feeding. No changes were observed in (+/+) male and female mice on cholesterol-containing diets. As seen in Figure 5, levels of bilirubin and bile salts were significantly higher in female then in male mice, with bile salt levels reaching values as high as 1200 μM in female (-/-) mice fed reference diet + cholesterol. Dietary PC + cholesterol seemed to be associated with less pronounced increases in plasma bilirubin and bile salts in female (-/-) mice, but not in male (-/-) mice.

Mouse group	*Diet*	*Conjugated bilirubin µmol/L*	*Unconjugated bilirubin µmol/L*
Male +/+	**Ref**	bd	2.7 ± 1.2
	Ref + 0.25% chol	0.7 ± 1.2	4.0 ± 2.0
	PC	0.7 ± 1.2	2.7 ± 1.2
	PC + 0.25% chol	0.7 ± 1.2	4.7 ± 1.2
Female +/+	**Ref**	bd	4.0 ± 2.0
	Ref + 0.25% chol	0.7 ± 1.2	3.3 ± 1.2
	PC	bd	4.7 ± 1.2
	PC + 0.25% chol	0.7 ± 1.2	3.3 ± 1.2
Male -/-	**Ref**	2.0 ± 0.1 *	4.0 ± 2.0
	Ref + 0.25% chol	6.0 ± 0.1 *#	6.7 ± 1.2 #
	PC	2.0 ± 0.1	4.0 ± 0.1
	PC + 0.25% chol	4.0 ± 0.1 *#	8.0 ± 0.1 *#
Female -/-	**Ref**	0.7 ± 1.2	3.3 ± 1.2
	Ref + 0.25% chol	22.7 18.9 *#	32.7 ± 22.7 *#
	PC	1.3 ± 1.2	3.3 ± 1.2
	PC + 0.25% chol	8.7 ± 5.0 *#	18.7 ± 8.1 *#

bd = below detection limit

Table 3: Conjugated and unconjugated bilirubin in plasma of male and female (+/+) and (-/-) mice *fed reference (Ref), reference + cholesterol (Ref + 0.25% chol), phospholipid (PC) or phospholipid + cholesterol (PC + 0.25% chol) diet. Blood samples were taken at the end of the experiment by cardiac puncture as described in the material and method section. (*) significant difference mdr2 -/- versus mdr2 +/+, Mann-Whitney U test, $p < 0.05$, $n = 8$ per group. (#) significant difference dietary group versus other dietary groups, one-way ANOVA, Newmann-Keuls t-test $p < 0.05$, $n = 8$ per group.*

Table 4 shows ASAT, ALAT and AP activities in male and female (+/+) and (-/-) mice on the four experimental diets. In male and female (-/-) mice there was an increased activity of all three enzymes compared with sex-matched controls when fed non-cholesterol containing diets. Feeding cholesterol had no further effect on ASAT and ALAT in male and female (+/+) and (-/-) mice, whereas there was a two to four-fold increase in AP activity in male and female (-/-) mice on cholesterol-containing reference diet.

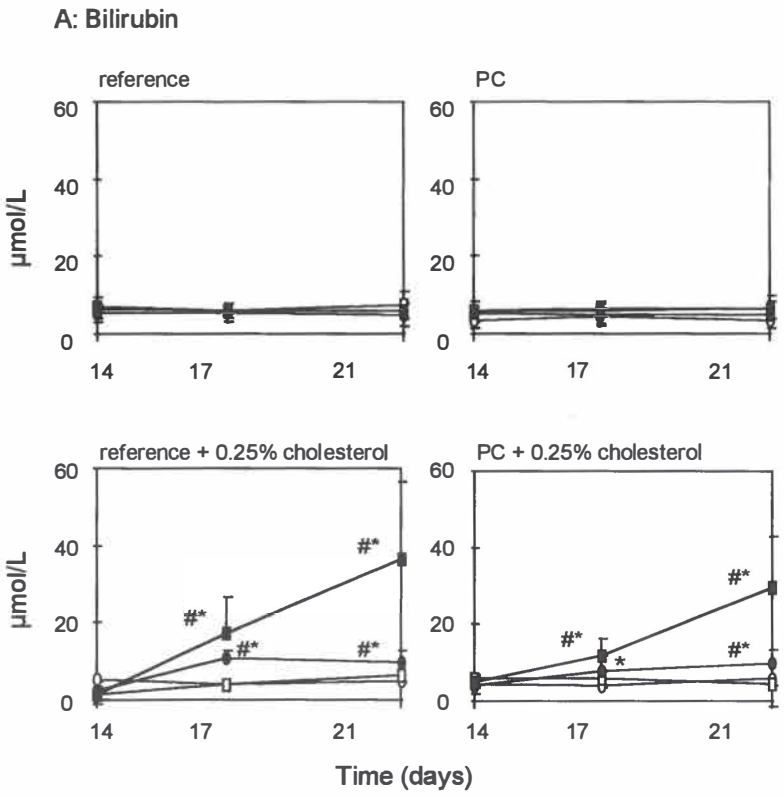

Figure 5a. Plasma bilirubin (panel A) and bile salt (panel B) levels in mice on reference, *reference + 0.25% cholesterol, phospholipid (PC) and phospholipid + 0.25% cholesterol (PC + 0.25%) diet during cholesterol feeding, i.e., at days 14, 17 and 21. Values represent mean ± SD in µmol/L, n = 8. Differences between dietary groups were determined by means of an one-way ANOVA with Newmann-Keuls t-test posthoc analysis, (#) p < 0.05. Differences between (-/-) and (+/+) mice was analyzed for each dietary group and males and females separately by Mann-Whitney U-test, (*) p < 0.05.* ❍ *mdr2 (+/+) male mice,* □ *mdr2 (+/+) female mice,* ● *mdr2 (-/-) male mice and* ■ *mdr2 (-/-) female mice*

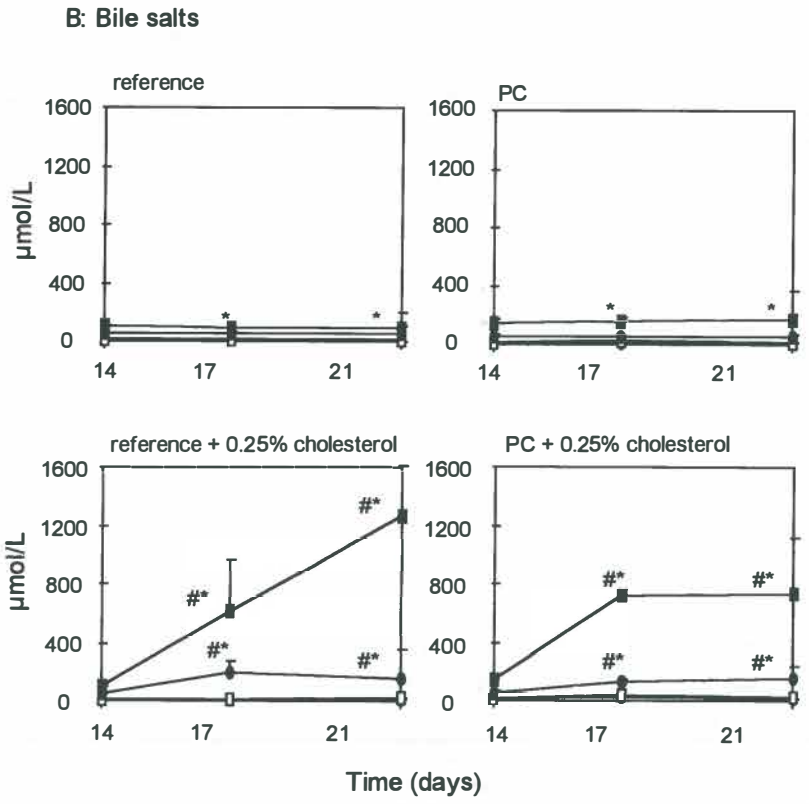

Figure 5b. Plasma bilirubin (panel A) and bile salt (panel B) levels in mice on reference, *reference + 0.25% cholesterol, phospholipid (PC) and phospholipid + 0.25% cholesterol (PC + 0.25%) diet during cholesterol feeding, i.e., at days 14, 17 and 21. Values represent mean ± SD in µmol/L, n = 8. Differences between dietary groups were determined by means of an one-way ANOVA with Newmann-Keuls t-test posthoc analysis, (#) $p < 0.05$. Differences between (-/-) and (+/+) mice was analyzed for each dietary group and males and females separately by Mann-Whitney U-test, (*) $p < 0.05$.* ❍ *mdr2 (+/+) male mice,* □ *mdr2 (+/+) female mice,* ● *mdr2 (-/-) male mice and* ■ *mdr2 (-/-) female mice*

Mouse	*Diet*	*cholesterol (mM)*	*cholesterol esters (mM)*	*triglycerides (mM)*	*Phospholipids (mM)*	*Free fatty acids (mM)*
Male +/+	**Ref**	4.1 ± 0.2	3.2 ± 0.3	0.6 ± 0.2	2.0 ± 0.3	1.8 ± 0.3
	Ref + 0.25%	4.4 ± 0.2	3.2 ± 0.6	0.7 ± 0.2	2.6 ± 0.1	2.2 ± 0.2
	PC	4.3 ± 0.3	3.2 ± 0.2	0.7 ± 0.2	2.9 ± 0.4 #	1.6 ± 0.5
	PC + 0.25%	5.4 ± 0.5 #	4.3 ± 0.2 #	1.0 ± 0.5	3.0 ± 0.5 #	1.9 ± 0.3
Female +/+	**Ref**	3.3 ± 0.4	2.4 ± 0.1	0.8 ± 0.2	1.7 ± 0.1	1.8 ± 0.7
	Ref + 0.25%	3.4 ± 0.6	2.5 ± 0.3	0.4 ± 0.2 #	1.8 ± 0.1	1.5 ± 0.4
	PC	3.3 ± 0.3	2.8 ± 0.2 #	0.6 ± 0.2	2.0 ± 0.6	1.9 ± 0.7
	PC + 0.25%	3.9 ± 0.4 #	3.1 ± 0.2 #	0.8 ± 0.3	2.0 ± 0.6	1.7 ± 0.5
Male -/-	**Ref**	2.1 ± 0.5 *	1.9 ± 0.2 *	0.8 ± 0.2 *	2.1 ± 0.8	2.1 ± 0.5
	Ref + 0.25%	1.9 ± 0.2 *	1.5 ± 0.3 *	0.6 ± 0.1	1.9 ± 0.4 *	1.3 ± 0.3 * #
	PC	2.3 ± 0.4 *	2.0 ± 0.1 *	1.1 ± 0.2 * #	2.5 ± 0.4	2.0 ± 0.1
	PC + 0.25%	2.7 ± 0.2 * #	1.9 ± 0.1 *	1.1 ± 0.3 #	2.1 ± 0.3 *	1.8 ± 0.2
Female -/-	**Ref**	1.9 ± 0.3 *	1.6 ± 0.2 *	0.8 ± 0.3	2.5 ± 0.3	2.1 ± 0.2 #
	Ref + 0.25%	2.0 ± 0.6 *	1.0 ± 0.4 *	1.3 ± 0.7 *	1.5 ± 0.4	1.2 ± 0.5
	PC	1.5 ± 0.3 *	1.2 ± 0.2 *	0.7 ± 0.2	1.9 ± 0.7	1.4 ± 0.5
	PC + 0.25%	2.0 ± 0.5 *	1.6 ± 0.5 *	0.9 ± 0.5	1.5 ± 0.2 *	1.2 ± 0.2

Blood samples were taken at the end of the experiment by cardiac puncture as described in the material and method section. (*) significant difference mdr2 -/- versus mdr2 +/+, Mann-Whitney U test, $p < 0.05$, $n = 8$ per group. (#) significant difference dietary group versus other dietary groups, one-way ANOVA, Newmann-Keuls t-test $p < 0.05$, $n = 8$ per group.

Table 4. *Alanine-aminotransferase (ALAT), aspartate-aminotransferase (ASAT) and alkaline phosphatase (AP) activity in plasma of male and female (+/+) and (-/-) mice fed reference (Ref), reference + cholesterol (Ref + 0.25% chol), phospholipid (PC) or phospholipid + cholesterol (PC + 0.25% chol) diet*

Bile flow and bile composition: The increases in plasma bilirubin and bile salt levels in the (-/-) mice on the cholesterol-containing diet could be indicative for induction of cholestasis. Therefore, we analyzed bile flow and biliary output of bile salts, bilirubin, cholesterol and phospholipid in the different groups, as summarized in Figure 6. As previously shown (8), bile flow in (-/-) mice is clearly increased compared with (+/+) mice, both in males and females. Although expected on basis of the elevated plasma bile salts, there was no decrease in bile flow in cholesterol-fed (-/-) mice compared to control fed mice. Interestingly, biliary bile salt output was not changed in cholesterol-fed male or female (-/-) mice compared with mice fed control diets and no changes where observed in biliary bilirubin output. Feeding cholesterol led to an increased biliary cholesterol output in (+/+) mice on both reference and PC diets. The cholesterol output in (-/-) mice was strongly impaired compared with the (+/+) mice (8,10) and did not show any change when PC and/or cholesterol were added to

the diet. Biliary phospholipid output was not changed in (+/+) mice of either sex in any of the dietary groups and remained below detection level in (-/-) mice (data not shown). Analysis of fecal acidic sterol content, performed in male animals only, revealed that feeding of cholesterol led to an approximately two-fold increased fecal bile salt output in (+/+) mice, i.e., from 6.5 ± 1 μmol/day and 5.7 ± 2 μmol/day in reference and PC-fed animals, respectively, to 12.2 ± 2 μmol/day and 14.7 ± 2 μmol/day in reference + cholesterol and PC + cholesterol-fed animals, respectively. In contrast, no increase in fecal bile salt output was noted in cholesterol-fed male (-/-) mice in which fecal bile salt output ranged from 6-9 μmol/day.

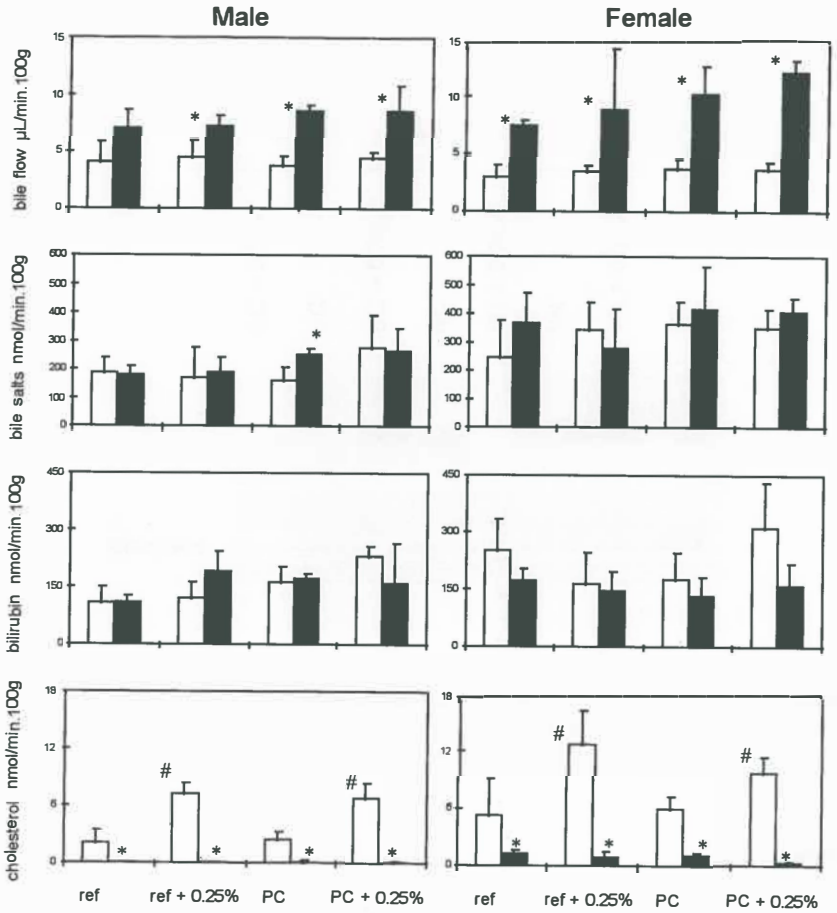

***Figure 6**: Bile flow, biliary salt output and biliary cholesterol output in male and female mice on reference (ref), reference + 0.25% cholesterol (ref + 0.25%), phospholipid (PC) and phospholipid + 0.25% cholesterol (PC + 0.25%) diet. Bile flow is given in μL per minute per 100g body weight, bile salt output and cholesterol output in nmol per minute per 100g body weight, n = 5 per group. Differences between the dietary groups were analyzed by one-way ANOVA analysis and Newmann-Keuls t-test posthoc analysis, (#) $p < 0.05$. Differences between (-/-) and (+/+) mice was analyzed for each dietary group and males and females separate by Mann-Whitney U-test, (*) $p < 0.05$. □ mdr2 (+/+) mice and ■ mdr2 (-/-) mice.*

Hepatic ntcp levels. In (-/-) mice on a regular chow diet, reduced levels of Na+-taurocholate cotransporting protein and of taurocholate transport in hepatic plasma membrane fractions have been found (NR Koopen et al., unpublished results). Therefore, we checked for ntcp protein levels in plasma membrane fractions isolated from livers of male and female (+/+) and (-/-) mice on the four experimental diets by Western blotting, as shown in Figure 7. The (-/-) mice showed a clearly decreased ntcp protein level compared with (+/+) mice when kept on reference diet. Addition of cholesterol to the reference diet caused a further decrease in ntcp levels in (-/-) mice, and also to decreased ntcp levels in (+/+) mice. PC feeding alone led to an apparent decrease in ntcp levels in (+/+) and (-/-) mice of both sexes. Addition of cholesterol to the PC diet did not have a clear additional effect in (-/-) mice, since the ntcp level was already low in the PC-fed (-/-) mice.

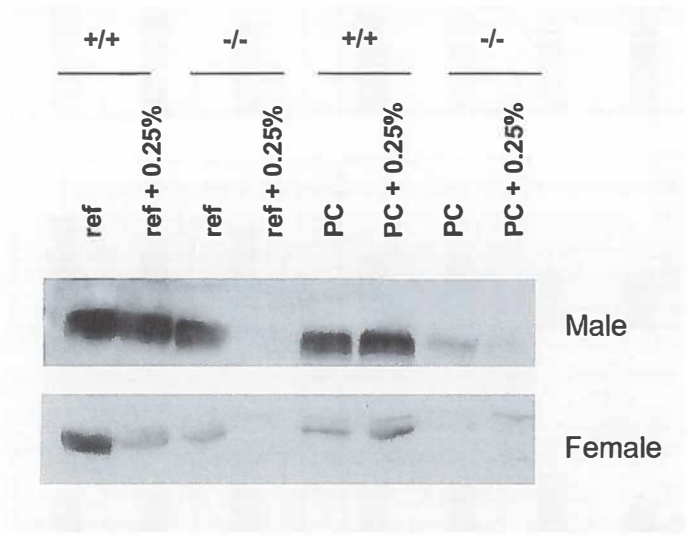

***Figure 7**: Western blot analysis of hepatic total membrane fractions of male and female mice for Na+-taurocholate cotransporting protein (ntcp). The amounts of protein loaded onto the gel were standardized to 75 μg per lane.*

DISCUSSION

Impaired biliary lipid secretion in the *mdr2* (-/-) mouse is associated with a strong decrease in plasma HDL cholesterol in presence of normal hepatic cholesterol levels. To investigate whether the decrease in plasma cholesterol levels in the (-/-) mouse is directly due to the impaired delivery of phospholipids and/or cholesterol to the intestine, we fed (-/-) mice diets supplemented with these lipids for a considerable period of time. The biliary pathway delivers approximately six to eight times more phospholipids and two to three times more cholesterol to the intestine of (+/+) mice than dietary

intake does, when animals are fed their regular lab chow. We have added phosphatidylcholine and/or cholesterol to the diet in amounts exceeding normal biliary delivery by a factor of approximately ten, to ensure the presence of sufficient amounts of these lipids in the intestinal lumen.

No changes were observed in plasma cholesterol, triglyceride, free fatty acid and phospholipid levels after feeding (+/+) and (-/-) mice diets supplemented with PC. This observation is not in line with data from Rioux et al. (26), who found a decrease in plasma cholesterol and triglyceride levels after feeding a phospholipid-enriched diet for a period of only four days to rats. These deviating results are possibly due to differences in duration of phospholipid feeding, phospholipid source and/or animal species. As shown in figure 2, plasma cholesterol transiently increased in (+/+) mice during reference + cholesterol and PC + cholesterol feeding, mainly in the HDL fraction, which is in agreement with findings of others (27). Analysis of feces showed a two-fold increase in fecal bile salt output both in reference + cholesterol and PC + cholesterol-fed (+/+) mice when compared with (+/+) mice fed reference or PC diets only, indicating increased hepatic bile salt synthesis. Figure 6 shows that after one week of cholesterol feeding in (+/+) mice there was almost a two fold increase in biliary cholesterol output. This is in line with earlier observations made in cholesterol-fed hamsters (28). The (+/+) mice apparently are capable to partly eliminate excess dietary cholesterol via these routes. In (-/-) mice, on the other hand, plasma cholesterol levels did not increase upon addition of PC and/or cholesterol to their diet. We analyzed hepatic cholesterol levels to assess whether dietary cholesterol was actually delivered to the liver in (-/-) mice. When expressed per liver, an equal increase in hepatic cholesterol content after cholesterol feeding in (+/+) and (-/-) mice was found. These results therefore indicate that dietary cholesterol must have been absorbed to a considerable degree in (-/-) mice, yet this influx of dietary cholesterol was not reflected in their plasma compartment. Increased fecal bile salt output nor increased biliary cholesterol secretion was found in (-/-) mice, indicating that these mice handle absorbed dietary cholesterol different than (+/+) mice do. Although the mechanism(s) underlying this differential handling of cholesterol remains to be elucidated, absence of any effect of PC and/or cholesterol feeding on plasma lipid levels indicates that reduced plasma cholesterol concentrations in (-/-) mice are not primarily caused by the absence of bile-type lipids *per se* in the intestinal lumen. This finding, therefore, suggests a more complex interrelationship between biliary and plasma lipids.

A surprising finding was the marked increase in plasma bile salt concentrations in (-/-) mice fed cholesterol. This, together with the also significant increase in plasma bilirubin levels and the elevated AP activities at first sight indicates induction of cholestasis by dietary cholesterol. However, no changes in bile flow and/or biliary bile salt and bilirubin output were induced by dietary cholesterol in (-/-) mice. Furthermore, no superimposed changes were induced in plasma ASAT en ALAT levels or on liver pathology (data not shown). Cholesterol feeding induced bile salt synthesis in (+/+) mice, as reflected in increased fecal bile salt output. This increase in fecal bile salt output was not reflected in increased

biliary bile salt output. The increase in fecal bile salt output was not seen in (-/-) mice. It has been described that excess feeding of cholesterol leads to increased bile salt loss from the intestine in rats (29) and humans (30) which has been attributed to a cholestyramine-like effect of dietary cholesterol. Since both (+/+) and (-/-) mice received the same diet containing 0.25% cholesterol the observed fecal bile salt loss in cholesterol-fed (+/+) mice can most likely not be attributed to such an effect. It should be noted that in the studie mentioned (29) diets containing 1% or 2% cholesterol were used while we used only 0.25% cholesterol containing diet. Bile collection was performed at the end of the seven day cholesterol feeding period while the fecal output was measured during the three days prior to the end of the experiment. Since plasma cholesterol tended to normalize during cholesterol feeding, biliary output may have already been adapted to this new steady state. Also, no changes were measured in mRNA levels of cholesterol 7 α-hydroxylase or cholesterol 27-hydroxylase, the two key enzymes in bile salt synthesis (data not shown). Increased mRNA levels of these enzymes have been described during feeding of 1% or 2% cholesterol in rats (29). Björkhem et al. (29) described that no changes in activity or mRNA levels are observed when feeding less than 1% cholesterol. A simple calculation, based on previously reported values (8), reveals that at least 3% and 15% of the bile salt pool resides in the plasma compartment in cholesterol-fed (-/-) male and female mice, respectively. It may be that a slightly induced bile salt synthesis rate/pool size exceeds the maximal transport capacity of bile salt secretion in (-/-) mice (8), leading to regurgitation in the plasma compartment. Increased plasma bile salt levels, also present during feeding of regular chow (8) and reference diet (this study), may down-regulate ntcp levels in the sinusoidal membrane, as proposed by Gartung *et al.* (31), thereby exacerbating the hypercholanemic response. Alternatively, down-regulation of ntcp may also reflect a physiological response to the cholesterol feeding *per se*, since such a down-regulation was also observed in (+/+) mice fed cholesterol that did not show elevated plasma bile salt levels. It may be that down-regulation of ntcp during cholesterol feeding functions to prevent feedback inhibition of bile salt synthesis by circulating bile salts, thereby facilitating conversion of excess cholesterol to bile salts. In fact, it has been shown that the promoter of ntcp in rats contains a sterol regulatory element (32), which could explain these findings and supports this hypothesis. The observation that effects of dietary cholesterol on plasma bile salt levels were more pronounced in female than in male (-/-) mice is in agreement with the fact that "basal" bile salt levels are already increased to a larger extent in the female (-/-) mice. The reason for this discrepancy between males and females is not yet clear, but may be related to the higher degree of liver pathology seen in females (11,12). Studies concerning the effects of chronic liver disease on ntcp expression and function are currently in progress in our laboratory. The effects of dietary cholesterol in (-/-) mice were not due to changes in bile salt composition, since no changes were found by gas chromatographic analysis of biliary bile salt composition in either (-/-) or (+/+) mice fed cholesterol (data not shown). Also in the PC-fed mice there was and reduced level of nctp protein. These results indicate that the effect of cholesterol may not be specific and that other lipids may also regulate this protein.

The increase in plasma unconjugated bilirubin found in (-/-) mice may be a consequence of high levels of circulating bile salts, exerting toxic effects on erythrocytes, which, in turn, leads to higher rates of hemoglobin release and bilirubin production. Bilirubin output in to bile was not affected in (-/-) mice fed cholesterol, which may indicate that biliary secretion in these animals already, under normal conditions, operates at maximal capacity. We speculate that the capacity of canalicular secretion of (conjugated) bilirubin in (-/-) mice is compromised due to reduced levels of mrp2, the major canalicular bilirubin transporter (33). Again, the effects observed were stronger in females than in males.

In conclusion, feeding of phospholipids and/or cholesterol at levels exceeding the physiological biliary input of these lipids about ten-fold does not normalize low plasma cholesterol levels in *mdr2* P-glycoprotein-deficient mice. Furthermore, cholesterol feeding of *mdr2* P-glycoprotein-deficient mice gives rise to "cholestatic" plasma levels of bilirubin and bile salts without evident changes in bile formation, compared to basal bile formation in these (-/-) mice. These "cholestatic" plasma levels may possibly related to down-regulation of hepatic uptake and/or canaliculair secretion systems. Down-regulation of ntcp during cholesterol feeding may be an aspecific physiological response, since PC feeding alone also lowers ntcp levels, to accelerate removal of excess cholesterol from the body by preventing feedback repression of hepatic bile salt synthesis. The results of this study imply that there is a specific role of biliary lipid secretion in the regulation of plasma lipoprotein levels.

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REFERENCES

1. Dietschy, J.M., Turley, S.D., and Spady, D.K. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. J.Lipid.Res. 1993;34(10):1637-1659.
2. Packard, C.J. and Shepherd, J. The hepatobiliary axis and lipoprotein metabolism: effects of bile acid sequestrants and ileal bypass surgery. J Lipid Res 1982;23(8):1081-1098.
3. Wilson, M.D. and Rudel, L.L. Review of cholesterol absorption with emphasis on dietary and biliary cholesterol. J Lipid Res 1994;35(6):943-955,.
4. Duckworth, P.F., Vlahcevic, Z.R., Studer, E.J., Gurley, E.C., Heuman, D.M., Beg, Z.H., and Hylemon, P.B. Effect of hydrophobic bile acids on 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity and mRNA levels in the rat. J Biol Chem 1991;266(15):9413-9418.
5. Spady, D.K., Stange, E.F., Bilhartz, L.E., and Dietschy, J.M. Bile acids regulate hepatic low density lipoprotein receptor activity in the hamster by altering cholesterol flux across the liver. Proc Natl Acad Sci 1986;6:1916-1920, 1986.

6. Lin, Y., Havinga, R., Schippers, I.J., Verkade, H.J., Vonk, R.J., and Kuipers, F. Characterization of the inhibitory effects of bile acids on very-low-density lipoprotein secretion by rat hepatocytes in primary culture. Biochem J 1996; 316(Pt 2):531-538.
7. Lin, Y., Havinga, R., Verkade, H.J., Moshage, H., Slooff, M.J., Vonk, R.J., and Kuipers, F. Bile acids suppress the secretion of very-low-density lipoprotein by human hepatocytes in primary culture. Hepatology 1996; 23(2):218-228.
8. Oude Elferink, R.P., Ottenhoff, R., van Wijland, M., Smit, J.J., Schinkel, A.H., and Groen, A.K. Regulation of biliary lipid secretion by mdr2 P-glycoprotein in the mouse. J.Clin.Invest. 1995;95(1):31-38.
9. Oude Elferink, R.P.J., Ottenhoff, R., van Wijland, M., Frijters, C.M.G., van Nieuwkerk, C.M., and Groen, A.K. Uncoupling of biliary phospholipid and cholesterol secretion in mice with reduced expression of mdr2 P- glycoprotein. J.Lipid.Res. 1996;37:1065-1075.
10. Smit, J.J., Schinkel, A.H., Oude Elferink, R.P., Groen, A.K., Wagenaar, E., van Deemter, L., Mol, C.A., Ottenhoff, R., van der Lugt, N.M., van Roon, M.A., van der Valk, M.A., Offerhaus, G.J.A., Berns, A.J.M., and Borst, P. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell. 1993;75(3):451-462.
11. Van Nieuwkerk, C.M.J., Oude Elferink, R.P.J., Groen, A.K., Ottenhoff, R., Tytgat, G.N.J., Dingemans, K.P., Weerman, M.A.V.B., and Offerhaus, G.J.A. Effects of ursodeoxycholate and cholate feeding on liver disease in FVB mice with a disrupted mdr2 P-glycoprotein gene. Gastroenterology. 1996;111:165-171.
12. Van Nieuwkerk, C.M.J., Groen, A.K., Ottenhoff, R., van Wijland, M., Weerman, M.A.V., Tytgat, G.N.J., Offerhaus, J.J.A., and Oude Elferink, R.P.J. The role of bile salt composition in liver pathology of mdr2 (-/-) mice: Differences between males and females. J.Hepatol. 1997;26:138-145 .
13. Tso, P. Intestinal lipid absorption. In: Physiology of the Gastrointestinal Tract, edited by Johnson, L.R.New York:Raven Press, 1994,p. 1867-1907.
14. Davidson, N.O., Kollmer, M.E., and Glickman, R.M. Apolipoprotein B synthesis in rat small intestine: regulation by dietary triglyceride and biliary lipid. J Lipid Res1986; 27(1):30-39.
15. Voshol, P.J., Havinga, R., Wolters, H., Ottenhoff, R., Princen, H.M.G., Oude Elferink, R.P.J., Groen, A.K., and Kuipers, F. Low plasma cholesterol and increased fecal sterol loss in mdr2 P-glycoprotein-deficient mice lacking biliary lipids. Gastroenterology 1998;114(5):1024-1034.
16. Navder, K.P., Baraona, E., and Lieber, C.S. Polyenylphosphatidylcholine decreases alcoholic hyperlipemia without affecting the alcohol-induced rise of HDL-cholesterol. Life Sci. 61(19):1907-1914, 1997.
17. Navder, K.P., Baraona, E., and Lieber, C.S. Polyenylphosphatidylcholine attenuates alcohol-induced fatty liver and hyperlipemia in rats. J Nutr 1997.;127(9):1800-1806.
18. Koopen, N.R., Wolters, H., Muller, M., Schippers, I.J., Havinga, R., Roelofsen, H., Vonk, R.J., Stieger, B., Meier, P.J., and Kuipers, F. Hepatic bile salt flux does not modulate level and activity of the sinusoidal Na+-taurocholate cotransporter (ntcp) in rats. J.Hepatol. 1997;27(4):699-706.
19. Stieger, B., Hagenbuch, B., landmann, L., Hochli, M., Schroeder, A., and Meier, P.J. In situ localization of the hepatocytic Na^+/Taurocholate cotransporting polypeptide in rat liver. Gastroenterology 1994.;107:1781-1787.
20. Twisk, J., Lehmann, E.M., and Princen, H.M. Differential feedback regulation of cholesterol 7 alpha-hydroxylase mRNA and transcriptional activity by rat bile acids in primary monolayer cultures of rat hepatocytes. Biochem J . 1993;290(Pt 3):685-691.
21. Bligh, E.G. and Dyer, W.J. A rapid method of total lipid extraction and purification. Can J Biochem Biophys 1995;191-917.
22. Kuipers, F., Van Ree, J.M., Hofker, M.H., Wolters, H., In t' Veld, G.I., Havinga, R., Vonk, R.J., Princen, H.M.G., and Havekes, L.M. Altered lipid metabolism in apolipoprotein E-deficient mice does not affect cholesterol balance across the liver. Hepatology. 1996.;24:241-247.
23. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.L. Protein measurement with the folin reagens. J Biol Chem 1951;193:265-275.
24. Murphy, G.M., Billing, B.H., and Baron, D.N. A fluorimetric and enzymatic method for the estimation of serum total bile acids. J Clin Pathol 1970;23:594-598.
25. Dawson-Saunders, B. and Trapp, R.G. Basic and clinical biostatistics, USA:Lange Medicals Books, 1990.
26. Rioux, F., Perea, A., Yousef, I.M., Levy, E., Malli, L., Carrillo, M.C., and Tuchweber, B. Short-term feeding of a diet enriched in phospholipids increases bile formation and the bile acid transport maximum in rats. Biochim Biophys Acta ,1994.;14(2):193-202
27. Iwata, T., Kimura, Y., Tsutsume, K., Furukawa, Y., and Kimura, S. The effects of various phospholipids on plasma lipoproteins and liver lipids in hypercholesterolemic rats. J Nutr Sci Vitaminol Tokyo 1993.;39(1):63-71.
28. Chen, J.M., Song, W., and Redinger, R.N. Effects of dietary cholesterol on hepatic production of lipids and lipoproteins in isolated hamster liver. Hepatology. 1996;24:424-434.

29. Bjorkhem, I., Eggertsen, G., and Andersson, U. On the mechanism of stimulation of cholesterol 7 alpha-hydroxylase by dietary cholesterol. Biochim Biophys Acta 1991;1085(3):329-335.
30. Duane, W.C. Effects of lovastatin and dietary cholesterol on sterol homeostasis in healthy human subjects. J Clin Invest 1993;92:911-918.
31. Gartung, C., Schuele, S., Schlosser, S.F., and Boyer, J.L. Expression of the rat liver Na+/taurocholate cotransporter is regulated in vivo by retention of biliary constituents but not their depletion. Hepatology. 1997;25:284-290.
32. Karpen, S.J., Sun, A.Q., Kudish, B., Hagenbuch, B., Meier, P.J., Ananthanarayanan, M., and Suchy, F.J. Multiple factors regulate the rat liver basolateral sodium-dependent bile acid cotransporter gene promoter. J.Biol.Chem. 1996;271:15211-15221.
33. Paulusma, C.C. and Elferink, R.P.J.O. The canalicular multispecific organic anion transporter and conjugated hyperbilirubinemia in rat and man. J.Molecular.Med-Jmm. 1997;75(6):420-428.

Chapter 7

Biliary Fibrosis Associated with Altered Bile Composition in a Mouse Model of Erythropoetic Protoporphyria

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Submitted

ABSTRACT

Background: Reduced activity of ferrochelatase in Erythropoetic Protoporphyria (EPP) results in protoporphyrin (PP) accumulation in erythrocytes and liver. Liver disease is a common complication in EPP. *Aim:* To investigate mechanisms underlying EPP-associated liver disease in a mouse model of EPP. Methods: Liver histology, indicators of lipid peroxidation, plasma parameters of liver function and bile composition were studied in mice homozygous (*fch/fch*) for a point mutation in the ferrochelatase gene, heterozygous (*fch*/+) and wildtype (+/+) mice. *Results:* Microscopic examination revealed bile duct proliferation and biliary fibrosis with porto-portal bridging in *fch/fch* mice. PP content (130-fold increased), TBARS (+30%) and conjugated dienes (+75%) were increased in *fch/fch* livers when compared with *fch*/+ and +/+, while hepatic thiols (-12%) and iron (-52%) were reduced. Liver enzymes and bilirubin levels in plasma were markedly increased in the homozygotes. Plasma bile salt levels were 80-times higher in *fch/fch* than in *fch*/+ and +/+ mice, probably related to the absence of the Na+-taurocholate cotransporting protein (ntcp) in *fch/fch* liver. Paradoxically, bile flow was not impaired and biliary bile salt secretion was 4-times higher in *fch/fch* mice than in controls. Fecal bile salt secretion was not increased: upregulation of the intestinal Na+-dependent bile salt transport system (ibat) in *fch/fch* mice may enhance efficiency of bile salt reabsorption. Bile salt-to-lipid ratio and PP content of *fch/fch* bile were respectively 2-fold and 85-fold increased relative to +/+, whereas biliary glutathione was reduced by 90%. Similar effects on bile formation were induced by griseofulvin-treatment of control mice. *Conclusion:* Impaired ferrochelatase activity strongly affects bile formation in mice. Rather than peroxidative processes, formation of cytotoxic bile with high concentrations of bile salts and PP may cause biliary fibrosis in *fch/fch* mice by inducing damage to bile duct epithelium.

INTRODUCTION

Erythropoietic protoporphyria (EPP) is an inherited disorder of heme synthesis caused by deficiency of the mitochondrial enzyme ferrochelatase (EC 4.99.1.1), responsible for insertion of ferrous iron into protoporphyrin (PP) (1). Reduced ferrochelatase activity in humans results in increased PP concentrations in erythrocytes, blood, liver and feces (1). Due to its hydrophobic nature, PP can only be removed from the body through secretion into bile followed by fecal elimination. Transport across the canalicular membrane appears to be rate-limiting in biliary PP secretion (2), but the transport systems involved have not yet been elucidated. It has been demonstrated that PP secretion in experimental animals is stimulated by bile salts (3, 4). Recent studies from our laboratory have shown that PP associates with biliary lipids and that the presence of cholesterol and phospholipids in bile is essential for efficient PP secretion (5). Hence, stimulation of lipid secretion may explain the reported stimulatory effects of bile salts on biliary PP secretion. When production of PP exceeds its utilization in heme synthesis and its maximal biliary excretion rate, progressive accumulation of PP in liver, erythrocytes and skin will occur.

Abnormal liver function tests are frequently observed in patients with EPP but the hepatic manifestations of the disease are diverse (1, 6). Up to 38 different mutations in the human ferrochelatase gene have been described sofar (7). It appears that only severe deficiencies in ferrochelatase activity predispose for development of liver disease, for which liver transplantation is indicated (6). Liver fibrosis progressing to cirrhosis develops in a considerable number of EPP patients, for instance in 7 out of 55 in a 20-years follow-up study by Doss and Frank (8). Biliary fibrosis has also been described in a mouse model of EPP (9) in which the homozygous presence of a point mutation in the ferrochelatase gene (*fch/fch*) leads to a reduction of enzyme activity by 95%. Development of liver damage in EPP has been ascribed to toxic actions of PP, due to its peroxidative properties, and to precipitation of (poorly soluble) PP in bile canaliculi (6). Although plasma bilirubin and liver enzymes are elevated in *fch/fch* mice (9), it is not known to what extent the bile formation process is actually affected in these animals. This is of importance to know, since recent studies in mdr2 P-glycoprotein-deficient mice have shown that the formation of cytotoxic bile *per se* (without cholestasis) can lead to severe biliary fibrosis. In this particular animal model, in which phospholipid/cholesterol-free bile is being produced, the degree of liver pathology is related to the hydrophobicity of the bile salt pool (10-12). Because insight in the mechanism(s) leading to development of fibrosis is essential for prevention and treatment of this complication of EPP, we have investigated liver morphology in relation to indicators of hepatic lipid peroxidation as well as bile composition in ferrochelatase-deficient *fch/fch* mice.

MATERIALS AND METHODS

Mice. Male and female *fch/fch* and *fch/+* mice with a BALB/c background were generously supplied by dr. Xavier Montagutelli (Institut Pasteur, Paris, France) for establishing a breeding program at the Central Animal Laboratory, Faculty of Medical Sciences, University of Groningen. The animals were kept in a temperature- and light-controlled environment and were protected from direct light. Homozygous and heterozygous off-spring was individually phenotyped by plasma PP and bilirubin analysis. Age-matched control BALB/c mice were purchased from Harlan BV, Zeist, The Netherlands. Control animals were kept in the same environment and under the same ad libitum dietary conditions (RMH-B, Hope Farms BV, Woerden, The Netherlands) for at least 3 weeks prior to experiments. Animals recieved humane care according to local guidelines: experimental procedures were approved by the local Ethical Committee for Animal Experiments.

Experimental procedures. For the experiments described herein, only male mice of 12-14 weeks were used. Animals were anaesthetized by i.p. injection of Hypnorm (fentanyl/fluanisone) and diazepam and their gallbladders were cannulated for collection of bile as described previously (13). During surgery, livers were protected from UV light by means of a filter. During the 1 h bile collection period, animals were placed in an humidified incubator to ensure maintenance of body temperature. After bile collection, a large blood sample (0.8-1.0 mL) was collected by cardiac puncture and immediately transferred to an EDTA-containing tube for separation of erythrocytes and plasma. Erythrocytes were washed with phosphate-buffered saline (PBS) and stored for porphyrin analysis at –20oC until measurements. Livers were excised and weighed. Parts of the livers were stored in paraformaldehyde or frozen in liquid isopentane for microscopic evaluation. The remainders were snap-frozen in liquid nitrogen for biochemical analyses, isolation of a liver membrane fraction and RNA isolation. The small intestines of the mice were flushed with cold saline and frozen in liquid nitrogen for preparation of mucosal scrapings.

For determination of fecal and urinary bile salt output, *fch/fch* and +/+ mice (n = 4 in both groups) were housed in individual metabolic cages for 3 days. Feces and urine were collected daily and the quantities were determined by weighing, the first after freeze-drying. The 3 daily collections for each individual mouse were then pooled and carefully mixed before bile salt measurements (see below).

The same experimental procedures, except for feces and urine collection, were applied to male Swiss mice (Harlan BV, Zeist, The Netherlands) of 12-14 weeks fed either the control chow diet or the same diet to which griseofulvin (2.5% wt/wt) was added for a period of 2.5 weeks (14).

Microscopic evaluation. Liver tissue was fixed in 4% paraformaldehyde (vol/vol) and embedded in parafin. Sections were stained with Haematoxilin or Masson prior to evaluation.

Analytical procedures. Plasma alkaline phosphatase, ALT, AST and bilirubin were determined by routine clinical procedures. Plasma bile salts were measured enzymatically (15). PP and coproporphyrins I and III in erythrocytes, liver tissue and bile were determined by HPLC, as previously described (5). Conjugated dienes (16), TBA-reactive substances (17) and thiols (18) in liver homogenates were measured according to published methods. Total biliary bile salts, cholesterol and phospholipids were measured as previously described (13), the latter two after Bligh and Dyer extraction (19). Biliary, fecal and urinary bile salt composition were determined by capillary gaschromatography (13): the identity of some minor metabolites present in mouse bile was verified by gas chromatography-mass spectrometry on a Finnegan-MAT SSQ combination (Finnegan MAT, Bremen, Germany) and comparison to reported spectra. Hepatic and biliary iron contents were determined by Atomic Absorption Spectrometry.

Western blotting. A crude membrane fraction and a plasma membrane fraction was prepared from pooled mouse livers (n = 5-6 per group) and characterized according to Wolters et al. (20) for assessment of Na+-taurocholate cotransporting protein (ntcp) levels by Western analysis, exactly as previously reported (21). The anti-ntcp immunoglobulin G K4 was kindly provided by dr. Bruno Stieger, Zürich, Switserland.

Antisera against a 53-residue carboxyterminal portion of the rat (RIBMAL1) or human (HIBMAL2) intestinal Na+-dependent bile salt transporter (ibat/IBAT), fused at their aminoterminus to an E. Coli maltose binding protein moiety, were raised in guinea pigs (22). These antisera were used to assess ibat levels in crude liver membrane fractions and in homogenates and crude membrane fractions prepared from intestinal scrapings. For the latter purpose, the entire small intestine of +/+ and *fch/fch* mice was divided into four equal parts, opened longitudinally, and mucosae were scraped using a glass slide. Homogenates were prepared after 10-fold dilution (wt/wt) with NaHCO3 (1 mmol/L, pH 7.5) containing 17 mg/L phenylmethylsulfonylfluoride and a crude membrane preparation was isolated by centrifugation at 45.000 rpm and 4oC for 60 min in an Optima TM TLX table top ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). Pellets were resuspended in 250 mmol/L sucrose in 10 mmol/L Tris-HCl buffer (pH 7.5) with protease inhibitor cocktail Complete TM (Boehringer Mannheim, Mannheim, Germany, 1 tablet/50 mL) by passing 20 times through a 26.6 G needle. Liver crude membranes, homogenates of intestinal mucosa and crude membranes hereof (50-75 μg protein/lane) were loaded and run on a 4-20% polyacrylamide gel. Subsequently, the proteins were transferred to nitrocellulose (Amersham, Little Chalfont, UK) and immunoblotting was performed with the antisera at a 1:10.000 dilution. Immunodetection was carried out using a rabbit anti-guinea pig IgG antibody conjugated to HRP (Sigma, St. Louis, MO) at a 1:2.000 dilution. Detection was by the ECL Western blotting kit (Amersham) according to the manufacturer's instructions.

Northern blotting. Total RNA was isolated from livers according to Chromczynski (23). Determination of steady state mRNA levels for CYP7A and CYP27 was performed by Northern blotting, with hybridization conditions exactly as described (24). The 28S ribosomal RNA was used as an internal standard to correct for differences in amounts of total RNA. The mRNA levels were quantified by PhosphoImager (Fuji Fujix Bas 1000) by using the program TINA, version 2.08c.

Statistics. Comparison between data from +/+, *fch*/+ and *fch/fch* mice was done by ANOVA and post-hoc Newman-Keuls t-test. Differences between control and griseofulvin-treated animals were tested for significance by the Mann-Whitney U-test. A p value of <0.05 was considered significant.

RESULTS

Liver characteristics and morphology. Liver weight of *fch/fch* mice was higher than that of *fch*/+ and +/+ mice, which, in combination with an approximately 30% lower body weight, resulted in a significant increase of liver-to-body weight ratio in *fch/fch* when compared with *fch*/+ and +/+ mice, i.e., 0.118 ± 0.021, 0.063 ± 0.010 and 0.046 ± 0.004, respectively.

Microscopic examination of liver sections (Figure 1) revealed massive bile duct proliferation and biliary fibrosis in *fch/fch* mice only. Porto-portal bridging was frequently observed in these animals. PP deposits were present in small bile ducts of *fch/fch* livers. Except for the presence of abundant fat droplets, mainly in perivenous hepatocytes, no morphological abnormalities were detected in livers of *fch*/+ mice.

Hepatic PP content was 130-fold increased in *fch/fch* mice when compared to *fch*/+ and +/+ animals, as previously described (9), while the contents of coproporphyrins I and III were respectively 17-times and 25-times higher than control values, as summarized in table 1. No significant differences between *fch*/+ and +/+ mice in this respect were noted. The hepatic conjugated dienes and TBAR contents were slightly but significantly higher in *fch/fch* than in *fch*/+ and +/+ mice, whereas the total thiol content was somewhat lower. The hepatic iron content was reduced in both *fch/fch* and *fch*/+ mice when compared to +/+ mice.

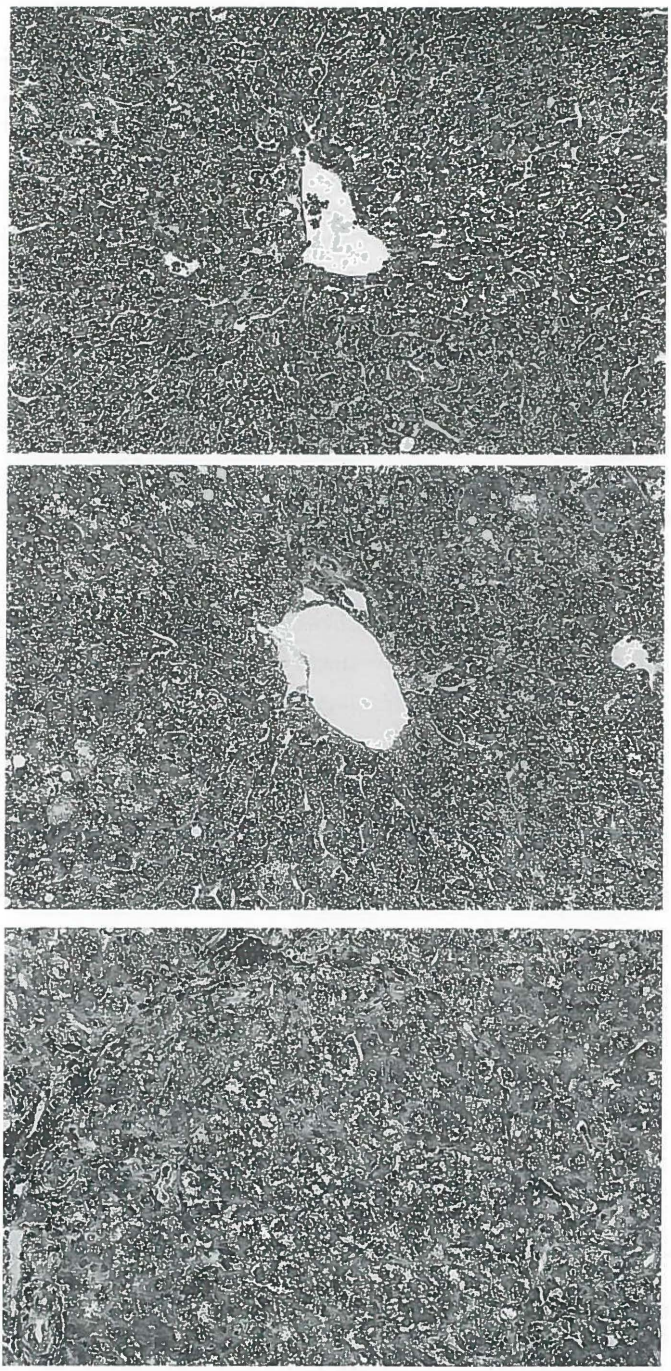

Figure 1: Representative sections of liver from wildtype (+/+), heterozygous (fch/+) or homozygous (fch/fch) mouse. Top: Portal triad of +/+ mice showing normal morphology. Middle: Perivenous fat accumulation is the only abnormality seen in fch/+ mice. Bottom: Severe bile duct proliferation and fibrosis in fch/fch mice, with protoporphyrin precipitation in bile ducts (brown). Original magnification 40x, Masson stain.

	+/+	*fch/+*	*fch/fch*
Protoporphyrin[a]	721 ± 593	506 ± 142	93018 ± 43696*
Coproporphyrin I[a]	4 ± 4	ND	71 ± 31*
Coproporphyrin III[a]	26 ± 26	16 ± 25	654 ± 214*
Conjugated dienes[b]	0.74 ± 0.07	0.68 ± 0.36	1.30 ± 0.09*
TBARS[c]	1.05 ± 0.13	121 ± 0.12	1.36 ± 0.07*$
Thiols[c]	78.6 ± 5.1	82.3 ± 7.8	69.1 P 11.5$
Iron	7.55 ± 1.31	5.49 ± 1.05	3.6 ± 0.44*$

*Mean values ±SD are shown for 6-7 mice per group. * indicates significant difference from +/+, $ indicates significant difference between fch/+ and fch/fch by ANOVA and post hoc Newman Keuls t-test. Values are given in apmoles/g liver, babsorbance 233/μmol lipid, cnmol/mg protein or dnmol/g liver. ND = non-detectable*

Table 1. Hepatic porphyrins, conjugated dienes, TBARS, thiols and iron content in wildtype (+/+), heterozygous (fch/+) and homozygous (fch/fch) ferrochelatase-deficient mice

Blood analyses. The PP contents of washed red blood cells were 21.0 ± 8.7, 2.0 ± 0.2 and 1.7 ± 0.1 μmol/L, respectively, for *fch/fch*, *fch/+* and +/+ animals. The difference between *fch/fch* mice on the one hand and *fch/+* and +/+ mice on the other hand is highly significant. These values are comparable to those previously reported (9).

Table 2 shows that alkaline phosphatase, ALT and AST levels in plasma of *fch/fch* mice were increased in comparison to *fch/+* and +/+ mice, as were the conjugated bilirubin and bile salt levels. Plasma bile salt concentrations up to 4700 μmol/L were measured in the *fch/fch* mice, i.e., values exceeding those seen after bile duct ligation in mice (900-1400 μmol/L after 14 days, R. Ottenhof, Amsterdam, personal communication).

	+/+	*fch/+*	*fch/fch*
AP[a]	21 ± 15	20 ± 10	327 ± 114*
AST[a]	80 ± 36	57 ± 19	1263 ± 777*
ALT[a]	53 ± 9	77 ± 37	1757 ± 876*
Bilirubin total[b]	9 ± 5	8 ± 3	152 ± 100*
Bilirubin direct[b]	8 ± 5	7 ± 3	150 ± 98*
Bile salts[b]	25 ± 8	36 ± 12	1819 ± 1610*

*Data represent mean values ± SD, n = 6 in all groups. *indicates significant diffeernce between fch/fch and fch/+ and +/+ mice by ANOVA. Values are given in aU/L and bμmol/L, respectively.*

Table 2: Plasma liver function parameters in wildtype (+/+), heterozygous (fch/+) and homozygous (fch/fch) ferrochelatase-deficient mice

	+/+	*fch/+*	*fch/fch*
Bile flow[a]	7.0 ± 1.3	9.4 ± 1.6*	9.7 ± 0.9*
Protoporphyrin[b]	2.8 ± 1.2	7.0 ± 1.9	243.3 ± 85*
Coproporphyrin I[b]	1.11 ± 0.24	1.28 ± 0.18	0.43 ± 0.52*
Coproporphyrin III[b]	3.38 ± 4.53	4.05 ± 2.53	1.97 ± 0.98*
Bilirubin[c]	0.35 ± 0.05	0.41 ± 0.13	0.76 ± 0.25*
Glutathione[c]	23.9 ± 7.3	53.1 ± 14.7*	3.7 ± 0.8*$
Bile salts[c]	185 ± 55	250 ± 63	910 ± 463*
Phospholipids[c]	13.2 ± 3.2	19.0 ± 4.7	27.7 ± 10.8*
Cholesterol[c]	2.5 ± 0.9	5.7 ± 1.7	10.3 ± 4.5*

*Values represent means ± Sd for n=6 per group. *indicates significant difference from +/+ values, $ indicates significant difference between fch/fch and fch/+ mice. Biliary output rates are given in aμL/min/100 g body weight, bpmol/min/100 g body weight, or cnmol/min/100 g body weight.*

Table 3: Bile flow and biliary secretion rates in wildtype (+/+) mice and in heterozygous (fch/+) and homozygous (fch/fch) ferrochelatase-deficient mice

Bile formation. Table 3 summarizes data on bile formation in the three strains of mice. In contrast to what was expected on the basis of plasma analysis, bile formation was not impaired in *fch/fch* mice. When expressed relative to body weight, bile flow was even higher in *fch/fch* and *fch/+* mice than in controls. The biliary secretion of PP was markedly higher in *fch/fch* mice than in the other two groups as was that of conjugated bilirubin. Biliary glutathione secretion, on the other hand, was reduced by 90% in *fch/fch* mice when compared with +/+ mice. Surprisingly, biliary bile salt secretion was markedly stimulated in the *fch/fch* mice. Increased bile salt output was accompagnied by an increased output of cholesterol and phospholipids, but the ratio of bile salts to lipids was clearly higher in *fch/fch* mice (mean bile salt-to-lipid ratio 24.0) than in *fch/+* (ratio 10.1) and in +/+ mice (ratio 11.8). Finally, biliary iron concentrations were below 3 μmol/L in all three groups.

Bile salt	+/+	*fch/+*	*fch/fch*
Total (mm/L)	26.3 ± 4.4 %	27.4 ± 7.9 %	86.4 ± 34.7* %
Deoxycholate	6.5 ± 0.8	6.7 ± 1.5	ND
Cholate	71.4 ± 2.7	62.7 ± 2.5*	45.2 ± 7.9 *$
CDC	1.7 ± 1.0	5.2 ± 1.7*	0.4 ± 0.2*$
β-Muricholate	13.0 ± 2.6	14.6 ± 1.0	41.4 ± 8.2 *
Δ 22-Muricholate	ND	ND	1.8 ± 0.5*
T-Muricholate	7.5 ± 1.1	10.9 ± 1.0*	11.2 ± 3.2*

*Data represent mean values ± SD, n = 5-6 in each group. * indicates significant difference from control values, $ indicates significant difference between fch/+ and fch/fch. CDC = chenodeoxycholate*

Table 4: Biliary bile salt concentration and composition in wildtype (+/+), heterozygous (fch/+) and homozygous (fch/fch) ferrochelatase-deficient mice.

Table 4 summarizes biliary bile salt composition in the three strains. The secondary bile salt deoxycholate could not be detected in bile of *fch/fch* mice and the relative contribution of its precursor, i.e., of cholate, was significantly reduced. The contribution of ß-muricholate, on the other hand, was significantly higher in *fch/fch* bile. In addition, Δ22-muricholate was detectable in bile of *fch/fch* mice but not in that of the other two groups.

To gain insight in mechanisms potentially underlying altered bile composition in ferrochelatase-deficient mice, we determined steady-state mRNA levels of cholesterol 7α-hydroxylase (CYP7A) and sterol 27-hydroxylase (CYP27), i.e., the enzymes catalyzing the initial steps in bile salt formation via the acidic and the neutral pathway, respectively. The mRNA levels of CYP7A and CYP27 tended to be higher in *fch/fch* (+126% and +170%, respectively) and *fch/+* (3% and 115%, respectively) mouse livers than in the control groups. Due to the large variations, however, only CYP7A mRNA levels in *fch/fch* mice differed significantly from those in *fch/+* and +/+ mice. However, subsequent studies revealed that these elevated hepatic CYP7A mRNA levels are not associated with increased bile salt synthesis in this particular animal model. Fecal bile salt excretion, under steady state conditions equal to hepatic synthesis rate, was similar in *fch/fch* mice (4.5 ± 3.1 μmol/day) and in +/+ controls (6.4 ± 3.9 μmol/day). In addition, urinary bile salt loss was significantly higher in *fch/fch* mice (174 ± 87 nmol/day) than in +/+ mice (18 ± 2 nmol/day), but more than an order of magnitude lower than the fecal loss.

Bile salt uptake systems is liver and intestine. To explain the cause of the exceedingly high plasma and biliary bile salt concentrations in *fch/fch* mice, membrane fractions were isolated for detection of Ntcp and ibat, responsible for Na+-dependent uptake of bile salts by the liver and the intestine, respectively. The Western blot shown in Figure 2A demonstrates that Ntcp is below detectable levels in the crude membranes and plasma membrane fractions isolated from *fch/fch* livers, in contrast to the situation in *fch/+* and +/+ membrane fractions.

In view of recent reports describing the presence of ibat and Na+-dependent taurocholate transport in bile duct epithelial cells (25, 26), the total liver membrane fractions were also probed for ibat. Figure 2B shows that ibat is detectable in +/+ membranes and present at higher levels in *fch/fch* membranes.

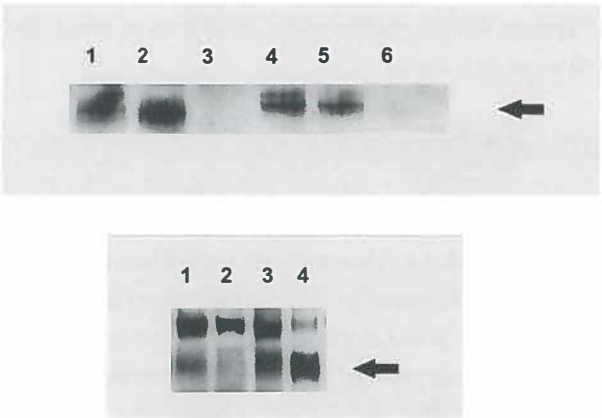

Figure 2: Western blots showing: A. Ntcp levels in total liver membrane fractions and plasma membrane fractions of wildtype mice (lanes 1 and 4, respectively), fch/+ mice (lanes 2 and 5, respectively) and fch/fch mice (lanes 3 and 6, respectively). Note the absence of Ntcp in fch/fch mice. B. Ibat levels in liver plasma membrane fractions of wildtype mice (lane 1), heterozygous fch/+ mice (lane 2) and homozygous fch/fch mice (lane 3), using the anti-rat ibat antibody (RIBMAL1). For comparison, a crude membrane fraction of rat ileum mucosa is shown in lane 4.

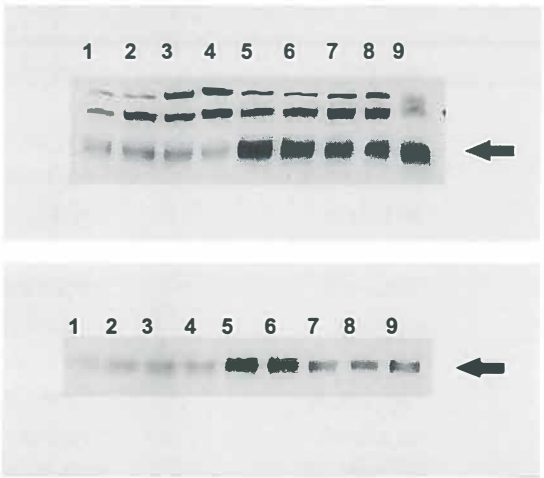

Figure 3: Western blot analysis of ibat levels in the small intestines of wildtype and fch/fch mice, using the anti-rat (RIBMAL1) (A) or the anti-human (HIBMAL2) (B) antibody, respectively. Small intestines of both groups of mice were divided into 4 equal parts, mucosae were collected and crude membrane preparations were prepared. Lanes 1-4 represent subsequent sections of wildtype mice, from proximal (lane 1) to distal (lane 4). Lanes 5-8 represent subsequent sections of fch/fch mice, from proximal (lane 5) to distal (lane 8). Lane 9 shows a crude membrane fraction of rat ileum for comparison. Localisation of ibat (~48 kD) is indicated by the arrow. When RIBMAL1 was used, additional bands were present, as also noted by others in rat intestine using different antibodies. These bands were not observed upon using HIBMAL 2.

Figure 3 shows that the levels of ibat are clearly increased in the upper segments of the small intestine of *fch/fch* mice, as detected both by the antisera raised against the rat (Figure 3A) and the human

(Figure 3B) protein. It appears that the gradient in ibat levels from proximal (low) to distal (high) in control mice is reversed in the *fch/fch* mice.

Bile formation in griseofulvin-induced EPP in mice. To ascertain whether or not the changes observed in bile formation are related to impaired ferrochelatase activity rather than to aspecific effects associated with the procedure employed to generate the mutation, a limited experiment was performed in which mice treated for 2.5 weeks with griseofulvin, a well-known inhibitor of ferrochelatase activity (27). Data summarized in table 5 clearly show that griseofulvin treatment leads to PP accumulation in red blood cells and in liver as well as to increased disposition of PP into bile. Furthermore, griseofulvin-treated mice showed an increase in liver weight and liver-to-body weight ratio, similar to the situation observed in ferrochelatase-deficient mice. Most importantly, plasma bile salt levels were only slightly elevated while bile flow and biliary bile salt secretion were markedly stimulated by griseofulvin. The bile salt-to-lipid ratio was increased from 11.9 to 25.9. The similarity between both independent models of impaired ferrochelatase activity supports the view that ferrochelatase-deficiency per se leads to altered bile composition.

	control (n)	*griseofulvin (n)*
Liver		
liver weight[a]	1.6 ± 0.2 (19)	2.9 ± 0.8* (8)
liver/body weight	0.05 ± 0.005 (19)	0.09 ± 0.01* (8)
Protoporphyrin[b]	398 ± 101 (3)	143000 (1)
Blood		
Protoporphyrin ery.[c]	1569 ± 510(6)	8714 ± 4847* (5)
Bile salt plasma[d]	46 ± 32 (6)	112 ± 98 (5)
Bile		
Bile flow[e]	5.28 ± 2.69 (19)	9.13 ± 1.31* (3)
Bile salts[f]	112 ± 46 (19)	244 ± 32* (3)
Cholesterol[f]	1.67 ± 0.80 (19)	2.95 ± 0.06* (3)
Phospholipids[f]	13.8 ± 6.2 (19)	8.3 ± 5.1 (3)
Protoporphyrin[g]	3.9 ± 3.2 (15)	1208 ± 181* (3)

*Data represent mean values ± SD of indicated number of animals. Values are given in ag, bpmol/g, cnmol/L, dμmol/L, eμL/min/100 g body weight, fnmol/min/100 g body weight, or gpmol/min/100 g body weight. *indicates significant difference between control and griseofulvin-treated mice*

Table 5: Effects of griseofulvin-treatment on liver weight, protoporphyrin levels and bile formation in Swiss mice

DISCUSSION

Liver fibrosis and cirrhosis as well as PP gallstones are frequently observed in humans with EPP. Acute liver failure requiring liver transplantation is much less common(6, 28). It has been noted that the development of EPP-associated liver disease may be related to the type of the disabling mutation: sofar, all known molecular defects in EPP patients with liver complications are either frame-shifts or nonsense mutations (7). Yet, there appears to be no clear-cut relation between the genetic defect on the one hand and PP accumulation, residual enzyme activity or severity of the disease on the other hand. Evidently, other factors contribute to the variable disease expression: Rüfenacht et al. (7) recently described clinical manifestations ranging from mild skin photosensitivity to terminal liver failure in four unrelated subjects carrying the same point mutation. In the present study, we have evaluated liver pathology in relation to bile composition in a mouse model of EPP generated during the course of a chemical mutagenesis experiment with ethylnitrosurea (9). Analysis of cDNA clones obtained from these mice revealed a T to A transition at nucleotide 293, leading to a methionine to lysine substitution at position 98 of the protein (29). Homozygosity for this mutation (*fch/fch*) reduces ferrochelatase activity in liver, spleen and kidney to 2.7%, 6.3% and 3.3% of control values, respectively (9). Reported enzyme activities in heterozygotes in these organs are 65%, 45% and 54% of control, respectively. Examination of livers obtained from 3 months-old *fch/fch* mice revealed portal inflammation and severe bile duct proliferation and biliary fibrosis with porto-portal bridging, associated with a marked enlargement of the liver. The hepatic PP content was dramatically increased in homozygous mutants and PP deposits were seen in bile ductules and in sinusoidal cells, presumably representing Kupffer cells. In contrast, no pathology was seen in livers of *fch*/+ mice, except for the presence of macrovesicular fat in perivenous areas. This indicates that 50% of normal enzyme activity, sufficient to maintain normal erythrocyte and hepatic PP levels, is also sufficient to prevent development of liver disease in these mice.

It has been suggested that so-called "dark effects" of PP, i.e., free-radical mediated damage initiated by PP in the absence of light, contributes to development of liver disease in EPP (30, 31). In the present study, we found indications for peroxidation in livers of *fch/fch* livers, i.e., increased conjugated dienes in the hepatic lipid fraction and elevated TBARS. Yet, the differences between *fch/fch* mice on the one hand and *fch*/+ and +/+ on the other hand were modest and relatively small when compared to those observed in established models of liver fibrosis and cirrhosis related to oxidant stress, like CCl4 and ethanol (32, 33). The low iron content of *fch/fch* livers may be beneficial in this respect, as iron has been shown to exacerbate oxidant stress-related fibrogenesis (34). To our opinion, therefore, it is unlikely that oxidant stress alone is responsible for the development of liver disease in *fch/fch* mice.

Persisting cholestasis *per se* can lead to irreversible liver damage. The bile duct ligated rat is a widely used model of biliary fibrosis progressing to cirrhosis (e.g., 35). Development of cirrhosis in this model

has been claimed to be preceeded by a complex sequence of events, involving toxic actions of bile salts on bile duct epithelium, chemokine and mitogen production by activated neutrophils and generation of reactive oxygen species. Based on plasma parameters, the *fch/fch* mice at first sight appear to be extremely cholestatic, with high liver enzymes, conjugated hyperbilirubinemia and particularly high bile salt levels. Surprisingly, however, bile formation appeared not to be compromized. On the contrary, bile flow and biliary bile salt secretion are higher in *fch/fch* mice than in controls, although it should be noted that the amount of fluid per mole of bile salt is reduced in *fch/fch* mice. The latter may be due to impaired bile salt-independent bile flow caused by the low biliary glutathione secretion. It could be argued that canulation of the gallbladder for collection of bile alleviates obstruction of the bile ducts by PP and thereby causes an enhanced flow of bile. However, there are a number of arguments against this option. First, obstruction of bile ducts is most likely to occur in smaller units inside the liver; this would not be solved by creating an outlet at the level of the gallbladder. Second, urinary bile salt loss is only 10-fold higher in *fch/fch* mice than in controls while bile duct ligation in rats leads to a persistent 4000-fold increase of urinary bile salts (36). Third, fecal bile salt output is not affected in *fch/fch* mice whereas a strong decrease would be expected in case of an obstruction (36). In addition, induction of ibat in the small intestine is consistent with an increased bile salt load in the intestine (37), although, obviously, other factors may also be involved herein. Fourth, release of bile duct obstruction is associated with an isolated hypersecretion of phospholipids into bile, at least in the rat (38): this is not seen in *fch/fch* mice. Finally and most importantly, short-term treatment of control mice with the ferrochelatase inhibitor griseofulvin stimulates bile flow and biliary bile salt secretion and leads to an increased bile salt-to-lipid ratio while, at the same time, plasma bile salts are not significantly elevated. This indicates that impaired ferrochelatase activity *per se* affects bile composition. Together, therefore, these observations eliminate frank biliary obstruction as the cause of fibrosis, although it can not be excluded that obstruction of some small bile ductules by PP precipitates does occur. However, we propose that bile duct proliferation and fibrosis are not due to the absence of bile formation but actually by the formation of excess bile with proliferation-inducing properties. Indeed, bile of *fch/fch* mice has a very high concentration of detergent bile salts that, on a molar basis, is accompagnied by less cholesterol and phospholipid than in *fch*/+ and in +/+ mice. Formation of "cytotoxic" lipid-free bile has recently been shown to underly the development of bile duct proliferation and biliary fibrosis in mdr2 Pgp-deficient mice (11, 12), i.e., a pathology similar to that observed in *fch/fch* mice. It is evident that in *fch/fch* mice the bile salt-to-lipid ratio is affected to a lesser extent than in mdr2 Pgp-deficient mice. In addition to strain differences that may influence susceptibility of the biliary epithelium, the presence of other bile components in high concentrations, such as PP and coproporhyrins, and the absence of others, such as glutathione, may contribute to the overall cytotoxic potential of *fch/fch* bile towards the epithelial cells.

At the moment, we can only speculate about the causes of abnormalities in bile formation induced by ferrochelatase-deficiency. High bilirubin output rates probably reflect accellerated hemoglobin

turnover, while low biliary glutathione may be an indicator of the compromized thiol status of liver cells and/or reduced mrp2/cmoat activity (39). The latter may also contribute to conjugated hyperbilirubinemia in *fch/fch* mice. Of particular interest, however, are the overt changes in bile salt metabolism. The limited experiment performed with griseofulvin-treated mice shows that pharmacological inhibition of ferrochelatase activity leads to similar changes in bile salt metabolism in mice as ferrochelatase-deficiency does, indicating that the observed effects are indeed related to impaired enzyme activity. Bile salt secretion is increased in *fch/fch* mice with proportionally more muricholates and no secondary bile salts. Fecal and urinary bile salt analysis revealed that hepatic bile salt synthesis is not increased in *fch/fch* mice but actually tends to be decreased. The finding that hepatic mRNA levels of CYP7A are increased in *fch/fch* mice therefore most likely has no physiological meaning and may indicate that activity of this cytochrome P450-dependent enzyme is limited by the availability of heme. Why then is biliary bile salt output increased in *fch/fch* mice and are, at the same time, plasma bile salt levels extremely elevated ? The latter is, in all likelyhood, related to the absence of Ntcp in the liver. Whether this is a cause or a consequence, as proposed by Gartung et al. (40), of high plasma bile salts can obviously not be concluded at this point. We speculate that high biliary bile salt concentrations, not accompagnied by sufficient lipids, and high PP concentrations damage cholangiocytes and/or obstruct small bile ductules. This will induce bile duct proliferation leading to an increased overall ibat activity in the bile ducts and to increased reabsorption of bile salts at this level. These bile salts are thus retained in the liver and return to the hepatocytes for resecretion into bile. Such an "intrahepatic bile salt circulation" may lead to increased bile salt concentrations in the hepatic circulation and inside hepatocytes and, by spill-over, to increased concentrations in the systemic circulation. Increased intracellular bile salt concentrations may down-regulate Ntcp, thereby aggrevating bile salt accumulation in the systemic circulation. The increased concentration of bile salts in the bile due to intrahepatic circulation results in an enhanced flux into the intestine, leading to upregulation of ibat, even in the upper parts of the intestine. Induction of the ibat system accellerates enterohepatic cycling of the bile salts. The presence of an intrahepatic circulation and a short-circuit of the enterohepatic circulation, the latter supported by the absence of deoxycholate in the pool, causes a very high biliary bile salt secretion rate and a dysbalance between bile salt and lipid output (41), leading to the formation of cytotoxic bile and further proliferation of bile duct cells that progresses to biliary fibrosis. Clearly, this proposed sequence of events is highly speculative but the various aspects of this hypothetical scheme can be approached experimentally.

In conclusion, ferrochelatase-deficient mice develop severe biliary fibrosis progressing to cirrhosis within 3 months. Development of liver disease is associated with the formation of cytotoxic bile: the similarity in this respect between the *fch/fch* mice and the mdr2 Pgp-deficient mice, i.e., two independent models with altered bile composition, suggest that changes in bile composition in general may be important in initiation of biliary liver disease.

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REFERENCES

1. Kappas A, Sassa S, Galbraith RA, Nordmann Y. In: Scriver CR, Beadet AL, Sly WS, Valle D, eds. The metabolic basis of inherited disease. New York: McGraw-Hill, 1989: 1305-1365.
2. Avner DL, Berenson MM. J Lab Clin Med 1982; 99: 885-894.
3. Berenson MM, Garcia-Marin JJ, Gunther C. AmJ Physiol 1988; 255: G382-388.
4. Berenson MM, el-Mir MY, Zhang LK. Am J Physiol 1995; 268: G754-763.
5. Beukeveld GJJ, In 't Veld G, Havinga R, Groen AK, Wolthers BG, Kuipers F. J Hepatol 1996; 24: 343-352.
6. Cox TM. J Inherit Metab Dis 1997; 20: 258-269.
7. Rufenacht UB, Gouya L, Schneider-Yin X, Puy H, Schafer BW, Aquaron R, Nordmann Y, Minder EI, Deybach JC. Am J Hum Genet 1998; 62: 1341-1352.
8. Doss MO, Frank M. Clin Biochem 1989; 22: 223-229.
9. Tutois S, Montagutelli X, Da Silva V, Jouault H, Rouyer-Fessard P, Leroy-Viard K, Guenet JL, Nordmann Y, Beuzard Y, Deybach JC. J Clin Invest 1991; 88: 1730-1736.
10. Smit JJM, Schinkel AH, Oude Elferink RPJ, Groen AK, Wagenaar E, van Deemter L, Mol CAAM, Ottenhof R, van der Lugt NMT, van Roon MA, van der Valk MA, Offerhaus GJA, Berns AJM, Borst P. Cell 1993; 75: 451-462.
11. Van Nieuwkerk CMJ, Oude Elferink RPJ, Groen AK, Ottenhof R, Tytgat GNJ, Dingemans KP, van den Bergh Weerman MA, Offerhaus GJA. Gastroenterology 1996; 111: 165-171.
12. Van Nieuwkerk CMJ, Groen AK, Ottenhof R, van Wijland M, van den Bergh Weerman MA, Tytgat GNJ, Offerhaus GJA, Oude Elferink RPJ. J Hepatol 1997; 26: 138-145.
13. Voshol PJ, Havinga R, Wolters H, Ottenhoff R, Princen HMG, Oude Elferink RPJ, Groen AK, Kuipers F. Gastroenterology 1998; 114: 1024-1034.
14. Preisegger KH, Stumptner C, Riegelnegg D, Brown PC, Silverman JA, Thorgeirsson SS, Denk H. Hepatology 1996; 24: 248-252.
15. Kuipers F, Havinga R, Bosschieter H, Toorop GP, Hindriks FR, Vonk RJ. Gastroenterology 1985; 88: 403-411.
16. Recknagel PO, Glende EA. Methods Enzymol 1984; 105: 331-337.
17. Ohkawa H, Ohishi N, Yagi K. Anal Biochem 1979; 95: 351-358.
18. Griffith OW. Anal Biochem 1980; 106: 207-212.
19. Bligh EG, Dyer WJ. Can J Biochem Biophys 1959; 37: 911-917.
20. Wolters H, Spiering M, Gerding A, Slooff MJH, Kuipers F, Hardonk M, Vonk RJ. Biochim Biophys Acta1991; 1069: 61-69.
21. Koopen NR, Wolters H, Muller M, Schippers IJ, Havinga R, Roelofsen H, Vonk RJ, Stieger B, Meier PJ, Kuipers F. J Hepatol 1997; 27: 699-706.
22. Kramer W, Wess G, Bewersdorf U, Corsiero D, Girbig F, Weyland C, Stengelin S, Ehnsen A, Bock K, Kleine H, Le Dreau MA, Schafer HL. Eur J Biochem 1997; 249: 456-464.
23. Chomczynski P, Sacchi N. Anal Biochem 1987; 162: 156-158.
24. Post SM, de Wit ECM, Princen HMG. Arterioscler Thromb Vasc Biol 1997; 17: 3064-3070.
25. Lazarides KN, Pham L, Tietz P, Marinelli RA, de Groen PC, Levine S, Dawson PA, LaRusso NF. J Clin Invest 1997; 100: 2714-2712.
26. Alpini G, Glazer SS, Rodgers R, Phinizy JL, Robertson WE, Lasater J, Caligiuri A, Tretjak Z, Le Sage GD. Gastroenterology 1997; 113: 1734-1740.
27. Yokoo H, Graig R, Haywood TR, Cochrane C. Gastroenterology 1979; 77: 1082-1087.
28. Sarkany RP, Alexander GJ, Cox TM. Lancet 1994; 343: 1394-1396.
29. Boulechfar S, Lamoril J, Montagutelli X, Guenet J, Deybach JC, Nordmann Y, Dailey H. Genomics 1993; 16: 645-648.
30. Koningsberger JC, Rademakers LH, van Hattum J, de la Faille HB, Wiegman LJ, Italiaander E, van Berge Henegouwen GP, Marx JJ. J Hepatol 1995; 22: 57-65.

31. Koningsberger JC, van Asbeck BS, van Hattum J, Wiegman LJ, van Berge Henegouwen GP, Marx JJ. Eur J Clin Invest 1993; 23: 716-723.
32. Tsukamoto H. Alcohol 1993; 10: 465-467.
33. Polavarapu R, Spitz DR, Sim JE, Follansbee MH, Oberley LW, Rahemtulla A, Nanji AA. Hepatology 1998; 27: 1317-1323.
34. Tsukamoto H, Horne W, Kamimura S, Niemela O, Parkkila S, Yla-Hertula S, Brittenham GM. J Clin Invest 1995; 96: 620-630.
35. Gross JB, Reichen J, Zeltner TB, Zimmermann A. Hepatology 1987; 7: 457-463.
36. Kunigasa T, Uchida K, Kadowaki M, Takase H, Nomura Y, Saito Y. J Lipid Res 1981; 22: 201-207.
37 Stravitz RT, Sanyal AJ, Pandak WM, Vlahcevic ZR, Beets JW, Dawson PA. Gastroenterology 1997; 113: 1599-1608.
38. Hardison WGM, Evans WH. J Hepatol 1986; 3: 318-327.
39. Muller M, Jansen PLM. Am J Physiol 1997; 272: G1285-1303.
40. Gartung C, Schuele S, Schlosser SF, Boyer JL. Hepatology 1997; 25: 284-290.
41. Groen AK, Oude Elferink RPJ, Tager JM. J Theor Biol 1996; 182: 427-436.

Chapter 8

Molecular mechanisms of cholestasis: causes and consequences of impaired bile formation.

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Submitted

INTRODUCTION

Hepatic bile formation and bile flow serve many important physiological functions. In the first place, cholesterol is excreted from the body almost exclusively via bile, either as the free compound or after conversion to bile salts. Therefore, bile formation is just as essential for maintaining cholesterol homeostasis as dietary intake and synthesis of the sterol, even though the latter processes have received much more attention. Secondly, biliary bile salts, in addition to being end-products of cholesterol catabolism, are essential for efficient biliary cholesterol secretion and for the solubilization of dietary fats in the intestine. Bile flow also has important toxicological and pharmacological ramifications. A large number of "waste products" of endogenous origin and xenobiotics are secreted into the bile, often after preceding oxidative or conjugative metabolism by hepatic phase I and phase II detoxifying systems, respectively. For instance, the majority of bilirubin is excreted in the form of its diglucuronide by the biliary route, and disturbance of any step in this process results in hyperbilirubinemia (jaundice) and the associated clinical manifestations. Heavy metals may serve as an example of toxic xenobiotics excreted into bile.

The biochemistry of bile formation has been focus of research in numerous laboratories, due both to its intrinsic mechanistic interest, relevant to fundamental principles of bioenergetics and membrane biochemistry, and to the serious clinical consequences resulting from disturbances of the process, i.e., cholestasis. Cholestasis, functionally defined as a cessation or impairment of bile flow, can lead to nutritional problems related to malabsorption of dietary fats and fat-soluble vitamins as well as to (irreversible) liver damage caused by accumulation of toxic compounds. Cholestasis is a frequently observed clinical condition; treatment, however, is often hampered by insufficient knowledge of underlying causes and difficulties in distinguishing the primary events from secondary consequences. On a mechanistic basis, cholestasis usually is divided into "extrahepatic" and "intrahepatic" forms. The first refers to obstruction of large bile ducts outside the liver, for instance due to gallstones. Bile duct ligation in rodents serves as an experimental model for this form of cholestasis. The causes of intrahepatic cholestasis lie within the liver, either at the level of the liver parenchymal cells, also known as hepatocellular cholestasis, or within intrahepatic bile ductules (cholangioles) and/or portal ducts. Experimental models exist for both, e.g. 17α-ethinylestradiol-cholestasis (1) for the first and sulfated glycolithocholate-cholestasis for the second form (2).

Generation of bile flow is an osmotic process driven by ongoing active secretion of solutes into the minute bile canalicular space between adjacent liver parenchymal cells (hepatocytes), that are sealed from blood by tight junctions, followed by passive influx of water and electrolytes by trans- or paracellular pathways. Conceptually, the liver can therefore be considered equivalent to a secretory epithelium, with the canalicular membrane corresponding to the apical membrane in a typical epithelium. Bile salts, in quantitative terms the major organic constituents of bile, are responsible for

generation of the main portion of bile flow in most species, including humans. This portion has been termed the bile salt-dependent fraction of bile flow (BSDF); the remainder, the bile salt-independent fraction (BSIDF), comprises the combined action of all other osmotically active bile constituents. Recent data indicate that glutathione is an important contributor to this fraction, at least in the rat (3,4) (Figure 1).

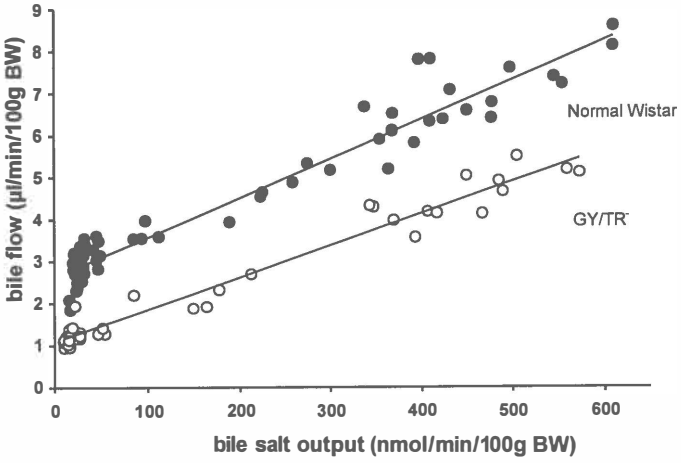

Figure 1. Relationship between bile flow and biliary bile salt secretion. *Rats were equipped with permanent catheters in bile duct and duodenum and both catheters were immmediately connected to each other to maintain the enterohepatic circulation. Eight days after surgery the enterohepatic circulation was interrupted and bile was collected in 30 min fractions, in both normal Wistar and mutant GY/TR⁻ Wistar rats. The bile flow at the hypothetical zero value of bile salt secretion represents the bile salt independent fraction of bile flow. This BSIDF is decreased in GY/TR⁻ rats, in a part caused by reduced biliary GSH secretion in these mutant rats.*

In the past couple of years it has become clear that the cells lining the bile ducts, the cholangiocytes, significantly contribute to bile formation by (secretin-stimulated) secretion of HCO_3^- and Cl^-. It has been estimated that cholangiocyte excretion accounts for up to 40% of human bile flow (5,6). In addition, the intrahepatic bile ducts represent an important target of injury in several disease states, such as primary biliary cirrhosis, primary sclerosing cholangitis and liver transplant rejection. For an extensive overview of the role of cholangiocytes in bile formation and cholestasis the reader is referred to recent review articles (7,8).

Bile salts are maintained in a so-called enterohepatic circulation, implying that, after being expelled into the intestinal lumen, these compounds are effectively reabsorbed and transported back to the liver via the portal system for uptake and re-secretion into the bile. Thus, in this case, as well as in the case of biliary secretion of other blood-bound compounds, three consecutive transport events occur and have to be considered: sinusoidal uptake, intracellular transport and canalicular secretion. Kinetic

analyses have revealed that, in most cases, the canalicular secretion step is rate-limiting for the overall process. Therefore, canalicular transport events are thought to constitute the molecular basis for bile production. In the past few years, a number of important canalicular transport systems have been identified and characterized (summarized in Table1). Most canalicular transport systems involved in bile formation are members of the ATP-binding cassette (ABC) transporter superfamily (9-12). Relevant transporters identified so far belong either to the P-glycoprotein (Pgp) or the Multidrug Resistance Protein (MRP) clusters of this superfamily (9,11). It is now recognized that absence or malfunction of specific transporters due to mutations in their encoding genes underlie specific, inherited forms of cholestatic liver disease. On the other hand, the regulation of these systems and their dysregulation as an underlying cause of disturbed bile formation is, at the moment, only marginally understood.

***Name*[1]**	***Species***	***Localization*[2]**	***M_r***	***Chromosome***
NTCP	human	BLM	50	14q24.1-24.2
ntcp	rat	BLM	51	6q24
oct1	rat	BLM	62	6q26
OATP	human	BLM	80	12q12
oatp1	rat	BLM	80	?
MDR1	human	CM	170	7q21
mdr1a	mouse	CM	150	5
mdr1b	mouse	CM	150	5
mdr1b	rat	CM	150	?
MDR3	human	CM	170	7
mdr2	mouse	CM	150	5
mdr2	rat	CM	150	5
SPGP	human	CM	170	2q24-36?
spgp	rat	CM	170	?
MRP1	human	LM	190	16p13.12-13
mrp1	mouse	LM	185	?
mrp2	rat	CM	200	?
MRP2	human	CM	200	10q24
MRP3	human	?	?	17q11-12
MRP5	human	?	?	3q25-26

Table 1. Properties of cloned hepatic transport proteins involved in bile formation. *Adapted from (9) [1]see text for full name. [2] BLM: basolateral plasma membrane; CM: canalicular plasma membrane. See http://www.med rug.nl/mdl/tab3.htm for regularly updated version.*

The aim of this review is to give a short overview of inborn errors and other conditions in humans leading to disturbed hepatobiliary transport and cholestasis. In combination with observations in experimental models related to these clinical states, these will be integrated to define the current state of knowledge about primary and secondary events in cholestasis.

2. Inborn errors of hepatobiliary secretion

In the past couple of years considerable progress has been made in characterization and cloning of hepatic transporters involved in bile formation and mutations affecting their transport activity have been identified. This knowledge has significantly contributed to our understanding of inherited cholestatic syndromes and provides the tools for detailed analysis of regulation of transport as well as for development of diagnostic procedures.

2.1 Bile salt transport

Efficient translocation of bile salts at the canalicular membrane is crucial in bile formation and maintenance of the BSDF. Processes involved in this important function of bile salts include their translocation through the hepatocyte, secretion into the bile canaliculus, reabsorption in the intestine and uptake from the portal blood at the sinusoidal membrane followed by resecretion into bile. In addition, hepatic bile salt synthesis is required to compensate for the small loss of bile salts via the feces to maintain an adequate bile salt pool size. Any disturbance in this complex sequence of events may underlie changes in BSDF.

Uptake systems: Uptake of bile salts by the hepatocytes is mediated by the Na^+-taurocholate cotransporting protein (ntcp) localized exclusively at the sinusoidal membrane (13,14). The *ntcp* gene contains a sequence identical to a bile salt responsive element present in the gene encoding cholesterol 7α-hydroxylase (*CYP7A*) (15,16) the rate-limiting enzyme in the bile salt synthesis, suggesting *ntcp* down-regulation by bile salts similar to the situation observed for *CYP7A*. Yet, variations in hepatic bile salt flux ranging from 0-300% of normal do not alter activity, protein levels or mRNA levels of ntcp in rats (17), indicating that this protein is constitutively expressed under non-cholestatic conditions. The uniform zonal distribution of ntcp (18) supports this view, since bile salts are normally transported mainly by periportal hepatocytes. On the other hand, *ntcp* is rapidly down-regulated at transcriptional and posttranscriptional level in experimental models of cholestasis, such as bile duct ligation (19), endotoxin administration (20-22) and ethinylestradiol treatment (23). This down-regulation has been ascribed to accumulation of bile salts in the hepatocytes: in fact, Gartung *et al.* (24) were able to demonstrate a significant negative correlation between plasma bile salt levels, presumably reflecting intracellular concentrations, and hepatic *ntcp* expression in rats. Similarly, *ntcp* is also rapidly down-regulated in cultured hepatocytes (25) and ntcp protein is virtually absent in hepatoma cell lines (25,26), i.e. cells experiencing dedifferentiation. Whether this downregulation is due to bile salts that accumulate in media of the cultured cells or to the dedifferentiation-related phenomena remains to be established. While high intracellular bile salt levels may be important for

regulation of *ntcp* under cholestatic conditions, recent data indicate that other factors are also involved. We were able to show that ethinylestradiol-cholestasis also leads to a strong reduction of ntcp levels in livers of long-term bile diverted rats (Figure 2).

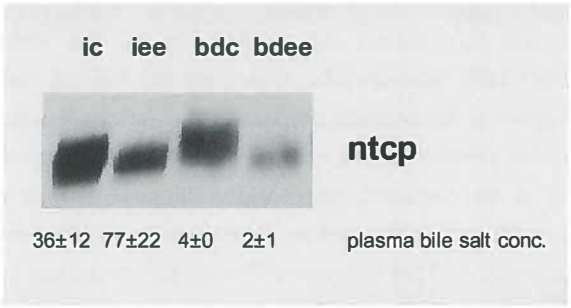

Fig 2. Ntcp levels in liver plasma-membranes isolated from EE treated control and 8 day-bile diverted rats.

Since in these rats the bile salt pool is depleted and plasma bile salts remain below detectable levels, it is highly unlikely that this effect is mediated by bile salts. Rather, it is likely that the estrogen itself or some factor related to the occurrence of cholestasis *per se* is involved. The consequences of absent or impaired ntcp activity is not yet clear. So far, functional mutations in *NTCP /ntcp* have not been reported and the generation of ntcp-deficient animals is eagerly awaited for. It should be noted that down-regulation of *ntcp* in EE-treated rats (23) and in ferrochelatase-deficient mice (unpublished results) is not associated with impaired biliary bile salt secretion. This may be due to overcapacity of the ntcp system or, alternatively, to the presence of other systems capable of bile salt uptake. Data available so far suggest that down-regulation of ntcp is a secondary effect of cholestasis that may be aimed at protection of the hepatocyte against the toxic actions of high intracellular bile salt concentrations. In this respect it is important to note that reconstitution of bile salt transport by transfection of cDNA encoding ntcp under the control of a cytomegalus virus in the MC Ardle RH-7777 cell line, that normally does not express *ntcp*, leads to profound structural changes and mitochondrial abnormalities when cells were incubated with physiological concentrations of bile salts (27).

Down-regulation during extrahepatic cholestasis in rats of the organic anion transporting polypeptide-1 (oatp1), a sodium-independent uptake system that also accommodates bile salts, may contribute to a decreased uptake of potential toxic bile salts in this situation (28). Alternatively, increased OATP mRNA in livers of patients with chronic cholestatic liver disease and upregulation of *OATP* expression in transfected HepG2 cells by low concentrations of taurocholate suggest that OATP may serve to enhance sinusoidal efflux of toxic compounds during cholestasis (29), as OATP has been shown to function bidirectionally as an anion exchanger (30).

Another important step in the maintenance of the enterohepatic circulation of bile salts involves their uptake from the intestine. While unconjugated bile salt can be reabsorbed passively to a certain extent, uptake of conjugated species is mediated by the ileal sodium-dependent bile salt transporter (IBST/ibst) (31,32). In contrast to ntcp (17), ibst appears to be regulated by physiological bile salt flux through the intestinal cells (33). Patients with mutations in *IBST* that affect function have been described. These patients suffer from diarrhea, steatorrhea and bile salt malabsorption, and, as a consequence of interruption of the enterohepatic circulation, they are thought to have a decreased bile salt poolsize (34). Two missense mutations have been identified in one allele of the *IBST* gene (also termed *SLC10A2*) at two conserved amino acid positions in a family with primary bile salt malabsorption. These mutations did not result in altered expression of the protein. Kinetic studies, however, revealed that transport of bile salts was defective when the mutant protein was transfected into COS cells (34). Interestingly, ibst has also been found in apical membranes of rat cholangiocytes, the cells lining the hepatic bile ducts (35,36). The physiological functions of the protein at this location remain to be revealed. It has been suggested that its presence enables an intrahepatic short-circuit for biliary bile salts, reminiscent of the cholehepatic shunt proposed by Hoffman and coworkers (37,38) for unconjugated bile salts. A major goal in the near future will be to establish the role of IBST in liver function during cholestasis, a condition frequently associated with marked bile duct proliferation.

Secretory systems. Functional studies in the early nineties revealed that there are two ATP-dependent systems involved in bile salt transport localized in the canalicular membrane (9). The first, recently identified as MRP2/mrp2 (39-41), accommodates bivalent, i.e. sulfated and glucuronidated bile salts that normally comprise only a small fraction of biliary bile salts. The other system accommodates monovalent, amino acid conjugated bile salts, for experimental purposes usually exemplified by taurocholate. The nature of this latter transporter, having a Km in the low micromolar concentration range, has not been clarified with certainty. It has been claimed, based on *in vitro* work, that the canalicular ecto-ATPase or C-CAM 105 could fulfill this function (42). However, strong arguments against this possibility have been put forward (43). Very recently, it has been claimed that a novel member of the P-gp family, originally cloned by Childs *et al.* (44), may actually be "the" bile salt transporter. This protein, called sister of P-glycoprotein (SPGP/spgp), is highly abundant in rat liver and exclusively present at the canalicular membrane (45,46). Most importantly, it has been shown in spgp-cRNA injected oocytes and in membranes isolated from Sf9 insect cells infected with a recombinant baculovirus containing spgp-cDNA, that spgp transports taurocholate in an ATP-dependent fashion. Kinetic analysis revealed that in these systems this taurocholate uptake is saturable with a Km value similar to that obtained in isolated canalicular membrane vesicles isolated from rat liver (46). Therefore, data available so far strongly indicate that spgp is the canalicular bile salt transporter. At this point, genetic studies in human cholestatic syndromes have recently provided important clues about the nature of bile salt transporting systems.

Progressive Familial Intrahepatic Cholestasis (PFIC). From genetic studies it is now clear that PFIC, a heterogeneous group of autosomal recessive cholestatic disorders characterized by severe fibrosis, cirrhosis and hepatitis progressing to liver failure before adulthood (47-52), consists of at least three different disorders with completely separated underlying causes. This insight has led to the proposal to distinguish between PFIC 1,2 and 3. (53). PFIC 1 and 2 appear to be associated with impaired bile salt transport capacity and are characterized by low γGT values in serum (51,52). In contrast, the defect in PFIC 3, characterized by high γGT levels in serum, has been pinpointed to defective phospholipid transport into bile (see below).

The first patients identified with PFIC-1 are descendants of Jacob Byler, an Amish settler from Pennsylvania; hence the "old" name Byler Disease (47). Positional cloning studies have located the genetic defect of this disease to chromosome 18q21-q22 (54). Interestingly, another type of inherited cholestatic disease, benign recurrent intrahepatic cholestasis (BRIC), was mapped to the same region of chromosome 18 (55). In contrast to the situation in PFIC-1, BRIC is characterized by recurrent attacks of cholestasis separated by symptom-free periods that may last from months to several years (56-58). Also in contrast to PFIC-1, BRIC is not associated with progressive liver damage (51,52,56-58). Very recent studies have provided evidence that the gene affected in PFIC-1 and BRIC (*FIC 1)* encodes for a P-type ATPase, showing homology with the microsomal aminophospholipid translocator (59). The gene appears to be expressed in the liver and, surprisingly, at an even higher level in the intestine. At the moment, the intracellular localization of the protein and its function are completely unknown. From the clinical presentation of both diseases it is likely that the protein is somehow involved in bile salt transport. In this respect it is important to note that the onset of cholestasis in BRIC is characterized by rapid increase in serum bile salt levels, before other cholestatic markers start to deviate (60). In addition, increased fecal bile salt loss due to intestinal malabsorption in non-symptomatic BRIC patients (61) is compatible with a function of the protein in bile salt transport in the intestine.

Patients with almost identical clinical presentation, but unrelated to the Byler family have been referred to as Byler Syndrome. In a number of these patients, the defect has also been mapped to chromosome 18, suggesting a similar etiology of the disease (62,63). Yet, Arnell *et al.* (64), who studied 8 Swedish families with PFIC, excluded linkage to 18q21-22, providing evidence for genetic heterogeneity in this disorder. Strautnieks *et al.* (53,65) studied 6 Middle East kindreds by homozygosity mapping and conventional linkage analysis, and mapped a locus for this subgroup to chromosome 2q24. As SPGP has recently been mapped to chromosome 2, it is attractive to speculate that the *SPGP* gene is affected in these patients with PFIC, now referred to as PFIC2 and that defective canalicular bile salt transport underlies this disease (66).

Inborn errors of bile salt synthesis. Disturbed *de novo* bile salt synthesis from cholesterol may contribute to decreased bile formation. A cascade of enzymatic conversions of the cholesterol

backbone leads via different pathways to the primary bile salts cholate and chenodeoxycholate (67,68). An incomplete conversion by improperly functioning enzymes in this route can result in the formation of toxic bile salt intermediates or bile salt metabolites that interfere with transport processes and, as a consequence, to cholestasis. At least five inborn errors of the bile salt biosynthetic pathways have been described (69,70).

The Smitz-Lemli-Opitz syndrome is characterized by extremely low levels of cholesterol in the circulation and very high concentrations of its precursor 7-dehydrocholesterol (71,72). These patients share a mutation in the gene encoding 7-dehydrocholesterol 7α-reductase, the last enzyme in the cholesterol biosynthetic pathway. Drastically reduced bile salt secretion in feces have been reported (71), indicating reduced bile salt synthesis. Although not directly measured to the best of our knowledge, it is highly likely that BSDF is impaired in these patients.

Progressive liver disease and cholestatic jaundice are seen in patients with a defect in 3β hydroxysteroid Δ5-dehydrogenase/isomerase (73-75). The di- en trihydroxy cholanoic acids and their sulfates that accumulate are unable to generate BSDF and inhibit biliary secretion of other compounds. It was shown that these atypical bile salts act as cholestatic agents by inhibiting the ATP-dependent transport system for bile salts (76). Deficiency for 3-oxo-Δ^4-steroid 5β-reductase leads to accumulation of 3-oxo-Δ^4-bile salts that are extremely hepatotoxic. These unusual bile salts were also shown to inhibit ATP-dependent bile salt transport (76). This, together with decreased amounts of primary bile salts that are formed result in a decreased BSDF (77,78). The gene encoding the latter enzyme has been cloned (79) and several mutations have been identified (70,79).

Cerebrotendinous Xanthomatosis is a rare lipid storage disease (80) associated with disturbed formation of bile salts, especially of chenodeoxycholate. Increased concentrations of so-called bile alcohols are found in these patients (69,81). This disease is caused by mutations in the *CYP27* gene, encoding the mitochondrial enzyme sterol 27-hydroxylase, involved in the formation of chenodeoxycholate via both the neutral and acidic pathways of bile salt synthesis. Several mutations in this gene have been identified so far (82-85). Finally, peroxisomal disorders are characterized by accumulation of dihydroxy and trihydroxy coprostanoic acids and their metabolites (86). Effects of these compounds on bile formation *per se* have not been reported.

In general, treatment with primary bile salts or with ursodeoxycholic acid may be beneficial in these patients, by improving the formation of BSDF and in case of primary bile salts, by suppressing synthesis of the atypical bile salts (87). Yet, in the case of a primary defect in bile salt transport, bile salt therapy is contraindicated by definition.

2.2 Phospholipid translocation

Secretion of phospholipids into bile is essential for efficient removal of the cholesterol in bile (88) and for protection of cells lining the bile ductuli and ducts against the actions of free (hydrophobic) bile salts. Biliary phospholipids consist mainly of phosphatidylcholine with bile-specific acyl-chain configuration (89). When phospholipid secretion is impaired, bile salts may disrupt membrane structures and membrane-bound enzymes appear in the circulation. Damage of epithelial cells leads to increased levels of γGT in the serum (90). The mdr2 Pgp localized in the canalicular membrane acts as an ATP-dependent phosphatidylcholine translocator or "flippase" (91,92). *Mdr2* knockout mice have been generated (88): these animals are unable to secrete phospholipids into bile. As a consequence, biliary cholesterol secretion is also severely impaired. However, bile flow and biliary bile salt secretion are not affected. Consequently, these mice are not cholestatic when the condition is defined on the basis of bile flow alone (88). Serum bile salt levels are increased in these animals (88), probably reflecting down-regulation of ntcp (93). The toxic action of bile salts at the level of the bile ducts leads to development of characteristic pathology, including bile duct proliferation and fibrosis. The severity of liver pathology could be influenced by modulation of the hydrophobicity of the bile salt pool in these mice. Ursodeoxycholate largely prevented progression of disease while cholate, a relative hydrophobic species, aggravated liver pathology (94).

MDR3 is the human homologue of the rodent mdr2 (95). Recently, inherited cholestatic liver disease in humans has been associated with MDR3 malfunction (96-98). This subtype of PFIC, proposed to be termed PFIC-3, is characterized by progressive cholestatic liver disease with features similar as described for the mdr2 Pgp-deficient mice associated with elevated γGT levels in serum (97). In a first paper a patient was described in which the *MDR3* mRNA could not be detected in biopsy material (97). More recently, analysis of genomic DNA encoding *MDR3* in two PFIC patients with high γGT revealed respectively a homozygous 7 bp deletion resulting in a frame shift, a premature stop codon and a nonsense mutation leading to a stop codon. Resulting truncated proteins may be rapidly degraded leading to absence of immunoreactive protein in the liver (99).

2.4 Organic anion transport

Secretion of organic anions into the canalicular lumen is mediated by an ATP-dependent organic anion transporting protein known as cmoat or mrp2. The human and rat genes encoding this transporter have been cloned (39,100). Rat strains lacking mrp2 (GY/TR$^-$ and EHBR) have generated insight into the function of this protein (101). These mutant rats show defective secretion of a broad range of organic anions including glutathione S-conjugates (102-105), reduced glutathione (105) glucuronidated and sulfated bile salts (106) and a variety of xenobiotics (102,107,108). Despite the absence of the mrp2, some organic anion transport activity is maintained in these rats, suggesting the presence of alternative pathways for secretion. Bilirubin-diglucuronide, for example, is secreted at virtually normal rates in these mutant rats albeit in the face of elevated serum and hepatic bilirubin

levels (101). This may be due to the presence of an electrogenic transporter for bilirubin diglucuronide (109). Other members of the ABC superfamily (110) and more specifically the mrp family (111) whose functions are not yet known, may contribute and/or compensate. Finally, a possible additional hepatic transporter has been partially characterized in functional terms but not yet sequenced (112,113). An assessment of its relationship to known transporters and its physiological role will have to await cloning.

The nature of the mutation in *mrp2* which inherits an autosomal recessive fashion has first been analyzed in GY/TR$^-$ rats by sequencing of the mutated *mrp2* after PCR amplification of its mRNA (39). A single base deletion was observed in the coding region, leading to an early stop codon and to formation of a truncated protein. In EHBR rats with a Sprague Dawley background, showing an identical phenotype as Wistar GY/TR$^-$ rats, another deletion was recently reported (100). The decrease in bile flow in these mutant rats is thought to be caused by the decreased secretion of GSH into the bile (102). In contrast to older reports (114), it was recently shown that mrp2 does mediate transport of reduced gluthatione in rats (105).

The recently cloned human MRP2 (41,115) is the homologue of the canalicular mrp2 of rat liver. In the human counterpart of the GY/TR$^-$ rat i.e., the Dubin Johnson patient, MRP2 is lacking (41). Recently, mutations in *MRP2* resulting in production of a truncated MRP2-protein in Dubin Johnson patients have been identified (40). Dubin Johnson patients show a mild conjugated hyperbilirubinemia, but like the mutant rat, do not have severe liver disease. Secretion of various organic anions has been found to be impaired in these patients (116).

Inborn errors in bilirubin conjugation. To allow efficient transport of bilirubin across at the canalicular membrane, glucuronidation of this poorly soluble compound is necessary. The glucuronidation process is mediated by the bilirubin-UDP-glucuronosyl transferase (B-UGT) (117,118). Defective glucuronidation leads to accumulation of unconjugated bilirubin in the liver and blood, manifesting as jaundice. The circulating high concentrations of unconjugated bilirubinin in these patients may cause neurological damage. Two diseases with defects in bilirubin conjugation are known. Crigler-Najjar type I patients lack the B-UGT caused by mutations in the conserved region of the gene (119). Milder forms of this disease, type II, are characterized by impaired activity of the enzyme. For both subtypes, several mutations are known (117). Patients suffering Gilbert Syndrome are homozygous for one additional TA in the TATAA sequence of the promotor region of the B-UGT gene. As a result, the gene is less efficiently transcribed, leading to a decreased expression of the enzyme and mild hyperbilirubinemia (120). The rat homologue for the Crigler Najjar Syndrome is the Gunn rat (121) that has a frame shift mutation leading to the formation of a truncated, inactive protein that is rapidly degraded (122).

3. Clinical condition associated with cholestasis

In addition to inborn errors causing cholestatic liver disease, there is a large number of clinical conditions associated with cholestasis. In this paragraph, some of these common clinical conditions and experimental models hereof are discussed.

3.1 Neonatal cholestasis

After birth, the neonate has to make some important shifts in its excretory pathways. The maternal placenta largely covers the removal of waste products from the fetus by specific transporters in the trophoblasts (123). After birth, this must be taken over by the newborn's liver. This change requires a certain adaptive period in which hepatic transport systems have to be adjusted, bile salt synthesis and bile salt pool have to be developed, and hepatic blood flow has to be redirected. Bile salt synthesis and bile salt conjugation in the fetus differ from those in adults as indicated by the atypical bile salts found in meconium and urine of newborns (124-126). As determined in rodents, BSDF is reduced in the neonatal state (127) and the BSIDF is completely absent (127). The latter is associated with a complete absence of glutathione in bile (3,128). This so-called physiological cholestasis usually spontaneously disappears when maturation of hepatic transport systems and bile salt metabolism has taken place. The ontogenetic development of hepatic transport systems has been studied mainly in rats. The oatp-1 mRNA levels appears at day 16 of gestation, remains stable until birth and then gradually increase in time (129). The ntcp mRNA, however, can first be detected at day 20 of gestation and levels gradually increase in time during further fetal and neonatal life (130). Recent studies on the developmental patterns of the canalicular organic anion transporter mrp2 and the bile salt transporter spgp showed a similar pattern. At gestation day 18-22 levels of mrp2 and spgp mRNA are about 2% of adult levels, then gradually increase during gestation and the postnatal period. Directly after birth the spgp levels are about 160% of adult levels and decrease to adult levels within weeks (131). Studies on bile formation during postnatal development shows a gradual increase in both BSDF and BSIDF (127). This is in agreement with the levels of respective transport proteins. In addition to the fact that the immaturity of biliary transport systems and hepatic metabolism cause physiological jaundice, it also renders the neonate extremely susceptible to various cholestasis-inducing factors, including total parenteral nutrition and sepsis (132,133). Surprisingly, however, the neonatal rat (134,135) and guinea pig (136,137) are extremely resistant towards the cholestatic actions of the secondary bile salt lithocholate. The underlying mechanism of this protection remains to be elucidated.

3.2 Total parenteral nutrition

Total parenteral nutrition (TPN) is associated with hepatic dysfunction and cholestasis in a considerable number of patients (132,133,138). Especially in young infants there is a high prevalence of TPN-cholestasis. Immaturity of the biliary system is probably an important factor contributing to hepatic problems associated with TPN in infants. Several mechanisms have been suggested to play a role. Absence of oral food intake and enteral stimulation affects the physiological stimuli for bile flow

generation and bile salt secretion (139,140) which, together with the reduced influx of bile salts from the intestine, results in a compromised enterohepatic circulation (133). The composition of TPN *per se* has been implicated. Solutions used for TPN contain glucose, amino acids and sometimes triglycerides. Both glucose and amino acids have been shown to decrease bile flow in animal models (141-144). Non-protein caloric overload has been shown to contribute to the incidence of cholestasis in TPN-treated patients (144-148). Other studies have implicated amino acids to be an important factor in the induction of cholestasis in humans (132,144). In one study, the effect of TPN on the development of cholestatic jaundice was tested in 82 infants. The volume of amino acids infused was related with the incidence of cholestasis, with the higher relative amounts of amino acids giving a higher prevalence (145). Another study pointed to a role for specific amino acids, in particular for methionine, as potential toxic agent (144). Deficiencies for specific amino acids have also been proposed (132). Since many of the TPN solutions given are hypertonic, TPN could give rise to cell volume regulatory responses in hepatocytes. Shrinkage of hepatocytes induced by hypoosmolarity of the perfusate has been shown to result in decreased transport of bile salts and to retrieval of mrp2 into pericanalicular vesicles (149-151).

Absence of intestinal stimulation during TPN leads to overgrowth of the small intestine with colonic bacteria. This enhances the intestinal production of lithocholate from chenodeoxycholate. Lithocholate administration leads to cholestasis with specific liver pathology in experimental animals (152,153). Similarities between TPN and lithocholate-induced histological changes in the liver suggest a relationship between the two entities (154,155). A role of secondary bile salts in the etiology of TPN-cholestasis is supported by studies demonstrating a decreased prevalence of cholestasis in patients treated with antibiotics (156-158). However, these results could not be confirmed in other studies (159).

3.3 Sepsis

Hyperbilirubinemia is frequently observed in septic patients (160-166). Hepatic handling of the organic anion bromosulphthalein (BSP) is altered in human volunteers injected with endotoxin (167) and in septic patients (166). In the latter study, canalicular excretion of the dye was found to be decreased. A reduced hepatic BSP clearance was also found in septic animal models (165,168-170). It was shown that the presence of *E. coli* endotoxin (LPS) in the perfusate of recirculating perfused rat livers gives rise to an acute reduction of the biliary excretion of BSP by 36%, accompanied by a decrease in both BSIDF and perfusate flow (171). Decreased glutathione secretion (-86%) and decreased HCO_3^- (-25%) secretion are probably responsible for this decrease in BSIDF (171,172). Both the basolateral uptake and canalicular secretion of the organic anions BSP and sulfated taurolithocholate, as measured in isolated perfused rat livers and in isolated basolateral and canalicular membrane vesicles from LPS-treated rats, were reduced (20). In the same study, it was shown that the maximal transport rate of bile salts was decreased by 60-80%. In basolateral membranes isolated from LPS-treated rats, Na^+ dependent taurocholate transport was decreased by about

40%. ATP-dependent bile salt transport measured in canalicular vesicles was decreased to an even greater extent (20,22). In both cases a decreased Vmax was found, indicating a reduction in the number of transporters available. These effects of endotoxemia are probably mediated by Tumor Necrosis Factor α (TNFα) (22,173): it was shown that the inhibition of bile salt transport induced by endotoxin is prevented, when rats are pretreated with anti-TNFα antibodies (173). In addition LPS, TNFα and interleukin-1β (IL-1β) were all shown to reduce *ntcp* mRNA levels (21). IL-6 did not affect ntcp mRNA, but taurocholate uptake in IL-6 incubated hepatocytes was reduced (21,174). This indicates that reduced basolateral bile salt uptake is caused by a decreased capacity of this uptake system. Furthermore, the activity of Na^+/K^+ATPase responsible for maintenance of a proper sodium gradient is decreased by LPS (175), which could contribute to the decreased bile salt uptake at the sinusoidal membrane.

Canalicular excretion of the mrp2 substrate, leukotriene D4 (LTD_4) is reduced by 80% in the endotoxemic treated rats (176). This is also the case for bilirubin-glucuronides (177). Down-regulation of mrp2 activity during endotoxemia appeared to be a gradual process with a maximal inhibition of 66% at 12 h after endotoxin-injection followed by a slow recovery during the subsequent 4 to 5 days (178). Treatment of rats with LPS resulted in a down-regulation of the mrp2 protein and mRNA levels (179). Protein levels of Pgp were not affected in this study (179). Furthermore, the inhibition of canalicular organic anion transport during endotoxemia can be counteracted by pretreating the animals with dexamethasone, an established inhibitor of cytokine production (178). LPS-induced cytokines are also potent stimulators of systemic and hepatic nitric oxide (NO) production. NO donors stimulate BSIDF in rat liver (178). In LPS-treated rats NO_2^- and NO_3^- levels were increased many-fold (178). However, both stimulation of NO synthesis with L-arginine and inhibition of NO production by aminoguanidine did not affect bile flow in LPS-treated rats, indicating that NO itself is not involved in disturbed bile formation during endotoxemia. If cytokines are the main mediators of the down-regulation of hepatobiliary transport during sepsis, this may also be of great importance for other situations were cytokines are produced, e.g., to explain impaired hepatobiliary transport in transplanted livers or in liver diseases like hepatitis and primary biliary cirrhosis.

3.4 Cholestasis of pregnancy

Intrahepatic cholestasis of pregnancy (ICP) is characterized by pruritis in the second half of pregnancy, sometimes associated with elevated serum bilirubin and transaminases (180-184). These symptoms generally disappear within a few hours to days after delivery. A geographic variation, with high incidences in Sweden and Chile, suggest a genetic component to be involved (181,183). This disease has been related to high perinatal mortality, high incidence of meconium staining, abnormal intrapartum heart rate and preterm deliveries (180,184). Patients susceptible for ICP often also develop cholestasis during the use of oral contraceptives (181). The actual mechanism causing ICP, however, has not been revealed yet. Genetic studies provide evidence that it is transmitted as

dominant trait which is sex-limited (182). However, males from families with a history of ICP show a decreased biliary secretion of BSP (185). This suggests that males can also transmit this trait to their female descendants (185). The increased frequency of ICP in mothers of patients with PFIC and BRIC (47,52,99,186) suggests that heterozygotes for mutations in transport systems involved in these diseases remain asymptomatic under normal circumstances, but cannot deal with increased pressure on these systems induced by pregnancy-related factors, e.g., elevated estrogen levels. Data available so far therefore suggest that mutations in different canalicular transport proteins and/or regulatory proteins may underlie ICP. Additionally, altered metabolism of estrogens leading to formation of cholestatic metabolites can not be excluded (187). The observations that slight elevations of serum bilirubin are present in 20% of normal pregnancies and that the secretion of BSP in ethinylestradiol (EE)-treated healthy volunteers is decreased, suggest that estrogens may also modulate canalicular secretion under 'physiological' circumstances (185,188).

Administration of EE to rodents is commonly used as a model for estrogen-related cholestasis. EE-treatment of rats mainly affects BSIDF as well as the maximum secretory rate for taurocholate into bile (1,189-191). Decreased fluidity of the sinusoidal membrane, decreased Na^+-K^+ ATPase activity and increased permeability of the tight junctions have been proposed as potential mechanisms (192-195). More recently, it was shown that Na^+ dependent sinusoidal transport and ATP-dependent canalicular transport of taurocholate in membrane vesicles isolated from EE-treated rats are decreased (196). Protein content and mRNA levels of ntcp were clearly decreased by EE (23). Both in rats and humans the maximal secretion rate of BSP is decreased by EE (179,185,196). In rats this is associated with decreased protein levels of mrp2 (179). The *mrp2* mRNA levels were not affected in EE-treated rats, indicating posttranscriptional regulation (179). We recently showed in mrp2-deficient GY/TR$^-$ rats that alternative pathways for biliary secretion of organic anions, including DBSP and bilirubin-diglucuronide, are also affected by EE, resulting in severe hyperbilirubinemia in GY/TR$^-$ rats upon EE treatment. This finding shows that EE exaggerates symptoms in situations in which canalicular transport is already affected (197). Besides canalicular organic anion transport, this could also be the case for other canalicular transport proteins such as spgp. It was shown that the transport maximum for both taurocholate and the organic anion BSP is decreased in neonatal rats born from mothers that underwent bile duct ligation during pregnancy (198). This again underlines the fact that special care has to be taken with children when maternal cholestasis occurs during pregnancy.

4. Cholestasis, causes and consequences

The mechanisms that underlie development of cholestasis in patients can be extremely varied. In its most simple form, mechanical obstruction of the biliary tree due to gallstones or hepatic or biliary tumors causes stagnation of bile flow. In the newly identified inborn errors in bile formation, for example in PFIC1 to 3, dysfunction of a crucial protein underlies impaired bile formation. At the other end of the spectrum, a complex interaction between subtle impairment of transport function,

accumulation of substrates and/ or their metabolites, damage to hepatocytes and subsequent inflammation may give rise to a viscious cycle leading to cessation of bile flow. In this situation, it is extremely difficult to distinguish primary events from secondary consequences. Yet, this distinction can be crucial for designing optimal treatment for the patient.

In animal models, a decrease in bile flow is, in general, associated with elevated cholestatic serum markers, altered liver histology and changes in sinusoidal and canalicular transport as determined *in vivo* and *in vitro* systems. The regulation of hepatic transport systems in cholestatic states has been and will be a major focus of research. However, a clear distinction between the mechanisms that actually cause cholestasis and the cascade of changes that is the result of cholestasis is also difficult to make in most experimental systems, including the model of bile duct ligation and recently generated knockout mice. For instance, extrahepatic cholestasis induced by bile duct ligation in rats causes bile duct proliferation, mitochondrial dysfunction, disruption of tight junction structure and redistribution of membrane proteins (199-206). In addition, down-regulation of canalicular-ABC transporters and of sinusoidal transporters like ntcp and oatp-1 occurs (19,179) and serum bile salts, bilirubin and transaminases increase. These changes are clearly secondary to induction of cholestasis. A number of these effects are also seen in intrahepatic cholestasis induced by EE. Yet, in this case, down-regulation of ntcp (23) and mrp2 (179) have been implicated in the onset of cholestasis. In our hands, administration of EE (5 mg/kg) for 3 consecutive days caused a 30% decrease in bile flow but did not, or only minimally, affect the serum markers of cholestasis in normal rats. However, even with this treatment schedule we found reduced mrp2 levels in the canalicular membrane and reduced biliary GSH secretion rates. In the same study, we found a very similar decrease in bile flow in GY/TR$^-$ rats that do not express *mrp2* and do not secrete GSH into bile (197). This example illustrates that a reduction of bile flow can be established without changes serum markers and that down-regulation of transporters are not always the cause of cholestasis but often a consequence. The down-regulation of ntcp in a number of cholestatic models probably provides a protective mechanism for the hepatocytes against intracellular accumulation of bile salts. Reversibly, serum bile salt levels and transaminases are increased and ntcp protein content and activity is decreased in *mdr2*-knock out mice, while bile flow is actually stimulated in these animals (Koopen *et al.* unpublished results). An even more striking example hereof is provided by ferrochelatase-deficient mice with deficient heme synthesis (207,208). In these mice, we recently found an 80-fold increase in serum bile salts, increased transaminases and bilirubin and an almost complete absence of ntcp protein in the liver. Paradoxically, bile flow in these animals is not decreased but increased. These findings illustrate very clearly that there may be differences between "clinical cholestasis" diagnosed on the basis of serum values of bile salts, bilirubin and transaminases and "actual" cholestasis, as defined by reduced bile flow. In this particular model, proliferation of bile ducts may modulate bile formation at non-hepatocytic level (7). Clearly, more research is needed to clarify the relationships between transporter activities, transporter localization (membranous versus vesicular) and the actual process of bile formation.

In conclusion, therefore, the identification and cloning of a number of key proteins involved in biliary transport and the description of common mutations herein, provide tools to diagnose the primary event in certain types of inherited cholestatic syndromes and allow for development of adequate treatment. For other, acquired types of cholestasis, treatment remains limited to palliative interventions until the real causes are defined.

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REFERENCES

1. Gumucio JJ, Valdivieso VD. Studies on the mechanism of the ethinylestradiol impairment of bile flow and bile salt secretion in the rat. Gastroenterology 1971;61:339-334.
2. Kuipers F, Hardonk MJ, Vonk RJ, Van der Meer R. Bile secretion of sulfated glycolithocholic acid is required for its cholestatic action in rats. Am J Physiol 1992;262:G267-G273.
3. Ballatori N, Truong A, T. Relation between the biliary glutathione excretion and bile acid-independent bile flow. Am J Physiol 1989;256:G22-G30.
4. Ballatori N, Truong A, T. Glutathione as a primary osmotic driving force in hepatic bile formation. Am J Physiol 1992;263:G617-G624.
5. Alavaro D, Mennone A, Boyer JL. Role of kinases and phosphatases in the regulation of fluid secretion and Cl^-/HCO_3^- exchange in cholangiocytes. Am J Physiol 1997;273:G303-G313.
6. Schlenker T, Romac JMJ, Sharara AI, et al. Regulation of biliary secretion through apical purinergic receptors in cultured rat cholangiocytes. Am J Physiol 1997;273:G1108-G1117.
7. Roberts SK, Ludwig J, La Russo N. The pathobiology of biliary epithelia. Gastroenterology 1997;112:269-279.
8. Marinelli RA, La Russo N. Solute and water transport pathways in cholangiocytes. Sem Liv Dis 1996;16:221-229.
9. Müller M, Jansen PLM. Molecular aspects of hepatobiliary transport. Am J Physiol 1997;35:G1285-G1303.
10. Gatmaitan ZC, Arias IM. ATP-dependent transport systems in the canalicular membrane of the hepatocyte. Physiol Rev 1995;75:261-275.
11. Dean M, Alkimets R. Evolution of ATP-binding cassette transporter genes. Curr Opin Genet Dev 1995;5:779-785.
12. Vore M. Canalicular transport: discovery of ATP dependent mechanisms. Toxicol Appl Pharmacol 1993;118:2-7.
13. Meier PJ. Molecular mechanisms of hepatic bile salt transport from sinusoidal blood into bile. Am J Physiol 1995;269:G801-G812.
14. Meier PJ, Eckhardt U, Schroeder A, Hagenbuch B, Stieger B. Substrate specificity of sinusoidal bile acid and organic anion uptake systems in rat and human liver. Hepatology 1997;26:1667-1677.
15. Stroup D, Crestani M, Chiang JYL. Identification of a bile acid responsive element in the cholesterol 7a-hydroxylase gene *CYP7A*. Am J Physiol 1997;273:G508-G517.
16. Karpen SJ, Sun AQ, Kudish B, et al. Multiple factors regulate the rat liver basolateral sodium dependent bile acid cotransporter gene promoter. J Biol Chem 1996;271:15211-15221.
17. Koopen NR, Wolters H, Müller M, et al. Hepatic bile salt flux does not modulate level and activity of the sinusoidal Na^+-taurocholate cotransporter (ntcp) in rats. J Hepatology 1997;27:699-706.
18. Stieger B, Hagenbuch B, Landmann L, Höchli M, Schroeder A, Meier PJ. In situ localization of the hepatocytic Na+/Taurocholate cotransporting polypeptide in rat liver. Gastroenterology 1994;107:1781-1787.
19. Gartung C, Ananthanarayanan M, Rahman M, et al. Down-regulation of expression and function of the rat liver Na^+/Bile acid cotransporter in extrahepatic cholestasis. Gastroenterology 1996;110:199-209.
20. Bolder U, Ton-Nu HT, Schteingart CD, Frick E, Hofmann AF. Hepatocyte transport of bile acids and organic anions in endotoxemic rats: impaired uptake and secretion. Gastroenterology 1997;112:214-224.
21. Green RM, Beier DR, Gollan JL. Regulation of hepatocyte bile salt transporters by endotoxin and inflamatory cytokines in rodents. Gastroenterology 1996;111:193-198.

22. Moseley RH, Wang W, Takeda H, et al. Effect of endotoxin on bile transport in rat liver: a potential model for sepsis-associated cholestasis. Am J Physiol 1996;271:G137-G146.
23. Simon FR, Fortune J, Iwahashi M, Gartung C, Wolkoff AW, Sutherland E. Ethinyl estradiol cholestasis involves alterations in expression of liver sinusoidal transporters. Am J Physiol 1996;271:G1043-G1052.
24. Gartung C, Schuele S, Schlosser SF, Boyer JL. Expression of the rat liver Na^+/ taurocholate cotransporter is regulated by in vivo retention of biliary constituents but not their depletion. Hepatology 1997;25:284-290.
25. Liang D, Hagenbuch B, Stieger B, Meier PJ. Parallel decrease of Na^+-taurocholate cotransport and its encoding mRNA in primary cultures of rat hepatocytes. Hepatology 1993;18:1162-1166.
26. Boyer JL, Ananthanarayanan M, Hofmann AF, et al. Expression and characterization of a functional rat liver Na^+ bile acid cotransport system in COS-7 cells. Am J Physiol 1994;266:G382-G387.
27. Torchia EC, Agellon LB. Bile-acid induced morphological changes in hepatoma cells with elevated sodium dependent bile acid uptake capacity. Eur J Cell Biol 1997;74:190-196.
28. Dumont M, Jaquemin E, D'Hont C, et al. Expression of the liver Na^+-independent organic anion transporting polypeptide (oatp-1) in rats with bile duct ligation. J Hepatology 1997;27:1051-1056.
29. Kullack-Ublick GA, Beuers U, Fahney C, Hagenbuch B, Meier PJ, Paumgartner G. Identification and functional characterization of the promoter region of the human organic anion transporting polypeptide. Hepatology 1997;26:991-997.
30. Shi XY, Bai S, Ford AC, et al. Stable induction expression of a functional rat liver organic anion transport protein in Hela cells. J Biol Chem 1995;270:25591-25595.
31. Kramer W, Girgig F, Gutjahr U, et al. Intestinal bile acid absorption. J Biol Chem 1996;268:18035-18046.
32. Wong MH, Oelkers P, Craddock AL, Dawson PA. Expression cloning and characterization of the hamster ileal sodium dependent bile acid transporter. J Biol Chem 1994;269:1340-1347.
33. Ananthanarayanan M, Cheng OI, Boyer JL, Suchy FJ. Characterization of cloned rat liver Na+-bile acid cotransporter using peptide and fusion protein antibodies. Am J Physiol 1994;267:G637-G643.
34. Oelkers P, Kirby LC, Dawson PA. Primary bile acid malabsorption caused by mutations in the ilieal sodium dependent bile acid transporter gene *(SLC10A2)*. J Clin Invest 1997;99:1880-1887.
35. Alpini G, Glaser S, Rodgers R, E.D., et al. Functional expression of the apical Na^+-dependent bile acid transporter in large but not small rat cholangiocytes. Gastroenterology 1997;113:1734-1740.
36. Lazaridis KN, Pham L, Tietz P, et al. Rat cholangiocytes absorb bile acids at their apical domain via the ileal sodium dependent bile acid transporter. J Clin Invest 1997;100:2714-2721.
37. Yoon YB, Hagey LR, Hofmann AF, Gurantz D, Michelotti JH, Steinbach JH. Effect of sidechain shortening on the physiologic properties of bile acids: hepatic transport and effect of biliary secretion of 23-nor-ursodeoxycholate in rodents. Gastroenterology 1989;90:837-852.
38. Gurantz D, Hofmann AF. Influence of bile acid structure on bile flow and biliary lipid secretion in the hamster. Am J Physiol 1984;247:G736-G748.
39. Paulusma C, Bosma PJ, Zaman GJ, et al. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. Science 1996;271:1126-1127.
40. Paulusma C, Kool M, Bosma PJ, et al. A mutation in the human canalicular multispecific organic anion transporter gene causes the Dubin Johnson syndrome. Hepatology 1997;25:1539-1542.
41. Kartenbeck J, Leuschner U, Mayer R, Keppler D. Absence of the canalicular isoform of the mrp gen-encoded conjugate export pump from the hepatocytes in Dubin -Johnson syndrome. Hepatology 1996;23:1061-1066.
42. Sippel CJ, Dawson PA, Shen T, Perlmutter DH. Reconstitution of bile acid transport in a heterologous cell by co-transfection of transporters for bile acid uptake and efflux. J Biol Chem 1997;272:18290-18297.
43. Kast C, Stieger B, Winterhalter KH, Meier PJ. Hepatocellular transport of bile acids. J Biol Chem 1994;269:5179-5186.
44. Childs S, Yeh RL, Georges E, Ling V. Identification of a sister gene to P-glycoprotein. Cancer Res 1995;55:2029-2034.
45. Müller M, Vos TA, Koopen NR, Roelofsen H, Kuipers F, Jansen PLM. Localization and regulation of a novel canalicular ABC-transporter. Hepatology 1996;24:368A(abst)
46. Gerloff T, Stieger B, Hagenbuch B, et al. The sister of p-glycoprotein represents the canalicular bile salt export pump of mamalian liver. J Biol Chem 1998;273:10046-10050.
47. Clayton RJ, Iber FL, Ruebner BH, McKusick VA. Byler Disease, fatal familial intrahepatic cholestasish in an Amish kindred. Am J Dis Child 1969;117:112-124.
48. Linarelli LG, Williams CN, Phillips MJ. Byler's disease: Fatal intrahepatic cholestasis. J Pediatr 1972;81(3):484-492.
49. Chobert MN, Bernard O, Bulle F, Lemonnier A, Guellaen G, Allagile D. High hepatic GGT activity with normal serum GGT in children with progressive idiopathic cholestasis. J Hepatology 1989;8:22-25.
50. Lachaux A, Loras-Duclaux I, Bouvier R, Dumontet C, Hermier M. Benign recurrent cholestasis with normal gamma-glutamyl-transpeptidase activity. J Pediatr 1992;121:78-80.

51. Alonso EM, Snover DC, Montag A, Freese DK, Whitington PF. Histologic pathology of the liver in progressive familial intrahepatic cholestasis. J Pediatr Gastroenterol Nutr 1994;18:128-133.
52. Whitington PF, Freese DK, Alonso EM, Swarzenberg SJ, Sharp HL. Clinical and biochemical findings in progressive familial intrahepatic cholestasis. J Pediatr Gastroenterol Nutr 1994;18:134-141.
53. Strautnieks SS, Kagawala AF, Tanner MS, et al. Identification of a locus for progressive familial intrahepatic cholestasis PFIC2 on chromosome 2q24. Am J Hum Genet 1997;61:630-633.
54. Carlton VHE, Knisely AS, Freimer NB. Mapping of a locus for progressive familial intrahepatic cholestasis (Byler disease) to 18q21-q22, the benign recurrent intrahepatic cholestasis region. Hum Mol Genet 1995;4:1049-1053.
55. Houwen RHJ, Baharloo S, Blankenship K, et al. Genome screening by searching for shared segments: mapping a gene for begign recurrent intrahepatic cholestasis. Nature Genetics 1994;8:380-386.
56. Summerskill JHJ, Walshe JM. Benign recurrent intrahepatic obstructive jaundice. Lancet 1959;2:686-690.
57. Shapiro RH, Isselbacher KJ. Benign recurrent intrahepatic cholestasis. New Engl J Med 1963;268:708-711.
58. Brenard R, Geubel AP, Benhamou JP. Benign recurrent intrahepatic cholestasis; A report of 26 cases. J Clin Gastroenterol 1989;11:546
59. Bull LN, van Eijk MJT, Pawlikowska L, et al. A gene encoding a P-type ATPase mutated in two forms of heriditary cholestasis. Nature Genetics 1998;18:219-224.
60. Bijleveld CMA, Vonk RJ, Kuipers F, Havinga R, Fernandes J. Benign recurrent intrahepatic cholestasis; a long-term follow-up study of two patients. Hepatology 1989;9:532-537.
61. Bijleveld CMA, Vonk RJ, Kuipers F, Koopman BJ, Wolthers BG, Fernandes J. Benign recurrent intrahepatic cholestasis: altered bile acid metabolism. Gastroenterology 1989;97:427-432.
62. Bull LN, van Eijk MJT, Liao M, et al. Genetics of PFIC-1 (Bylers Disease), a form of progressive familial intrahepatic cholestsasis. Hepatology 1997;26:369A(abst)
63. Tazawa Y, Abukawa D, Nakagawa N, et al. Progressive familial cholestasis linked to chromosome 18q21-q22 in a japanese family. Hepatology 1997;26:1022(abst)
64. Arnell H, Nemeth A, Anneren G, Dahl N. Progressive familial intrahepatic cholestasis (PFIC): evidence for genetic heterogeneity by exclusion of linkage to chromosome 18q21-q22. Hum Genet 1997;100:378-381.
65. Strautnieks SS, Kagawala AF, Tanner MS, Gardiner RM, Thompson RJ. Locus heterogeneity in progressive familial intrahepatic cholestasis. J Med Genet 1996;33:833-836.
66. Müller M, Jansen PLM. The secretory function of the liver: new aspects of hepatobiliary transport. J Hepatology 1998;28:344-354.
67. Vlahcevic ZR, Pandak WM, Heuman DM, Hylemon PB. Function and regulation of hydroxylases involved in bile acid biosynthesis pathways. Sem Liv Dis 1992;12:403-419.
68. Princen HMG, Post SM, Twisk J. Regulation of bile acid biosynthesis. Curr Pharm Design 1997;3:59-84.
69. Bjorkhem I. Inborn errors of metabolism with conseqences for bile acid biosynthesis. Scand J Gastroenterol 1994;29:68-72.
70. Setchell KDR, O'Connell NC. Inborn errors of bile acid biosynthesis: update on biochemical aspects. In: Fromm H, Leuschner U, eds. Bile acids- cholestasis-gallstones. Advances in basic and clinical bile acid research. Dortrecht/ Boston / London: Kluwer academic publishers, 1996:129-136.
71. Tint GS, Irons M, Elias E, Batta AK, Frieden R, Chen T. Defective cholesterol synthesis associated with the Smith-Lemi-Opitz syndrome. New Engl J Med 1994;330:107-113.
72. Irons M, Elias E, Salen G, Tint GS, Batta AK. Defective cholesterol biosynthesis in Smith-Lemli-Opitz syndrome. Lancet 1993;341:1414
73. Clayton PT, Leonard JV., Lawson AM, et al. Familial gint cell hepatitis associated with the synthesis of 3b 7a-dihydroxy- and 3b 7a12a-trihydroxy-5-cholanoic acids.
. J Clin Invest 1987;79:1031-1038.
74. Buchmann MS, Kvittingen EA, Nazer H, et al. Lack of 3b-hydroxy-delta5-C27-steroid dehydrogenase/isomerase in fibroblasts from a child with urinary excretion of 3b hydroxy-delta 5-bile acids. J Clin Invest 1990;86:2034-2037.
75. Jaquemin E, Setchell KDR, O'Connell NC, et al. A new cause of progressive intrahepatic cholestasis: 3b-hydroxy-C27-steroid dehydrogenase/isomerase deficiency. J pediatr 1994;125:379-384.
76. Stieger B, Zhang J, O'Neil B, Sjövall J, Meier PJ. Differential interaction of bile acids from patients with inborn errors of bile salt syntesis with hepatocellular bile acid transporters. Eur J Biochem 1997;244:39-44.
77. Setchell KDR, Suchy FJ, Welsh MB, Zimmer-Nechemias L, Heubi JE, Balistreri WF. delta 4-3-oxosteroid 5b-reductase deficiency described in identical twins with neonatal hepatitis-a new inborn error in bile acid biosynthesis. J Clin Invest 1988;88:2135-2146.
78. Clayton PT, Patel M, Lawson AM, Carruthers RA, Tanner MS, Strandvik B. 3-oxo-delta4-bile acids in liver direase. Lancet 1988;4:1283

79. Kondo K, Kai M, Setoguchi Y, Eggertsen G, Sjoblom P, Setoguchi T. Cloning and expression of cDNA of human delta 4 -3-oxosteroid 5b-reductase and substrate specificity of the expressed enzyme.
. Eur J Biochem 1994;219:357-362.
80. Oftebro H, Bjorkhem I, Skrede S, Schreiner A, Pedersen JL. CTX. A defect in mitochondrial 26-hydroxylation required for normal biosynthesis of cholic acid. J Clin Invest 1980;65:1418-1430.
81. Kim KS, Kubota S, Kurriyama M, Fujiyama J, Bjorkhem I, Eggertsen G. Identification of new mutations in sterol-27-hydroxylase in japanese patients with CTX. J Lipid Res 1994;35:1031-1039.
82. Cali JJ, Hsieh CL, Francke U, Russel DW. Mutations in the bile acid biosynthetic enzyme sterol 27-hydroxylation underlie CTX. J Biol Chem 1991;266:7779-7783.
83. Leitersdorf E, Reshef A, Meiner V, Revitzki R, Schwartz S, Dann EJ. Frameshift and splice-junction mutations in the sterol 27-hydroxylase gene cause CTX in Jews from Morrocan origin. J Clin Invest 1993;91:2488-2496.
84. Garuti R, Croce MA, Tiozzo R, et al. Four novel mutations of sterol 27-hydroxylase gene in Italian patients with cerebrotendinous xantomatosis. J Lipid Res 1997;38:2322-2334.
85. Chen W, Kubota S, Kim KS, et al. Novel homozygous and compound heterozygous mutations of sterol 27-hydroxylase gene (CYP27) cause cerebrotendinous xanthomatosis in three Japanese patients from two unrelated families. J Lipid Res 1997;38:870-879.
86. Kase BF, Pedersen JL, Strandvik B, Bjorkhem I. In vivo and in vitro studies on formation of bile acids in patients with the Zellweger syndrome. Evidence that peroxisomes are of importance in the normal biosynthesis of both cholic and deoxycholic acid. J Clin Invest 1985;76:2393-2402.
87. Setchell KDR, Balistreri WF, Piccoli DA, Clerici C. Oral bile acid therapy in the treatment of inborn errors of bile acid synthesis with liver direase. In: Paumgartner G, Stiehl A, Gerok W, eds. Bile acids as therapeutic agents. From basic science to clinical practice. Dortrecht: Kluwer, 1990:367-373.
88. Smit JJM, Schinkel AH, Oude Elferink RPJ, et al. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell 1993;75:451-462.
89. Coleman R, Rahman M. Lipid flow in bile formation. Biochim Biophys Acta 1992;1125:113-133.
90. Maggiore G, Bernard O, Lemonnier A, Allagile D. Diagnostic value of serum gamma-glutamyl-transpeptidase activity in liver diseases in children. J Pediatr Gastroenterol Nutr 1991;12:21-26.
91. Ruetz S, Gros P. Phosphatidylcholine translocase: a physiological role for the mdr2 gene. Cell 1994;77:1071-1081.
92. van Nieuwkerk CMJ, Groen AK, Ottenhoff R, et al. The role of bile salt composition in liver pathology of mdr2 (-/-) mice: differences between males and females. J Hepatology 1997;26:138-145.
93. Voshol PJ, Koopen NR, Havinga R, Oude Elferink RPJ, Groen AK, Kuipers F. Hepatic actions of dietary cholesterol in mdr2 P-glycoprotein-deficient mice. Hepatology 1997;26:294A(abst)
94. van Nieuwkerk CMJ, Oude Elferink RPJ, Groen AK, et al. Effects of ursodeoxycholate and cholate feeding on liver disease in FVB mice with a disrupted mdr2 P-glycoprotein. Gastroenterology 1996;111:165-171.
95. Smith AJ, Timmermans-Hereijgers JLPM, Roelofsen H, et al. The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. FEBS Lett 1994;263-266.
96. Dunia I, Smit JJM, van der Valk MA, Bloemendal H, Borst P, Benet LZ. Human multidrug resistance 3-P-glycoprotein expression in transgenic mice induces lens membrane alterations leading to cataract. J Cell Biol 1996;132:701-716.
97. Deleuze JF, Jaquemin E, Dubuisson C, et al. Defect of multidrug-resistance 3 gene expression in a subtype of progressive familial intrahepatic cholestasis. Hepatology 1996;23:904-908.
98. Jaquemin E, de Vree JML, Sturm E, et al. Mutations in the MDR3 gene are resposible for a subtype of progressive familial intrahepatic cholestasis. (PFIC). Hepatology 1997;26:284A(abst)
99. de Vree JML, Jaquemin E, Sturm E, et al. Mutations inthe MDR3 gen cause progressive familial intrahepatic cholestasis. Proc Natl Acad Sci 1998;95:282-287.
100. Ito K, Suzuki H, Hirohashi T, Kume K, Shimizu T, Sugiyama Y. Molecular cloning of canalicular multispecific organic anion transporter defective in EHBR. Am J Physiol 1997;272:G16-G22.
101. Jansen PLM, Peters W, H.M., Lamers WH. Heriditary chronic conjugated hyperbilirubinemia in mutant rats caused by defective hepatic anion transport. Hepatology 1985;5:573-579.
102. Oude Elferink RPJ, Ottenhoff R, Liefting W, de Haan J, Jansen PLM. Hepatobiliary transport of glutathione and glutathione conjugate in rats with hereditary hyperbilirubinemia. J Clin Invest 1989;84:476-483.
103. Büchler M, König J, Brom M, et al. cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. J Biol Chem 1996;271:15091-15098.
104. Keppler D, Leier I, Jedlitschky G. Transport of glutathione conjugates and glucuronides by the multidrug resistance proteins MRP1 and MRP2. Biol Chem 1997;378:787-791.

105. Paulusma C, van Geer M, Heijn M, Evers R, OttenhoffR, Oude Elferink RPJ. The canalicular multispecific organic anion transporter mediates transport of reduced glutathione. Hepatology 1997;26:292A(abst)
106. Kuipers F, Enserink M, Havinga R, et al. Seperate transport systems for biliary secretion of sulfated and unsulfated bile acids in the rat. J Clin Invest 1988;81:1593-1599.
107. Jansen PLM, Groothuis GMM, Peters W, H.M., Meijer DFK. Selective hepatobiliary transport of organic anions and neutral steroids in mutant rats with heriditary-conjugated hyperbilirubinemia. Hepatology 1987;7:71-76.
108. Oude Elferink RPJ, OttenhoffR, Radominska A, Hofmann AF, Kuipers F, Jansen PLM. Inhibition of gluthatione conjugate secretion from isolated rat hepatocytes by dipolar bile acids and other organic anions. Biochem J 1991;274:281-286.
109. Nishida T, Gatmaitan ZC, Roy-Chowdhry J, Arias IM. Two distinct mechanisms for bilirubin glucuronide transport by rat canalicular membrane vesicles. J Clin Invest 1992;90:2130-2135.
110. Alkimets R, Gerrard B, Hutchinson A, Dean M. bla bla. Hum Mol Genet 1996;5:1649-1655.
111. Kool M, de Haas M, Scheffer GL, et al. Analysis of expression of cMOAT (MRP2), MRP3, MRP4 and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1) in human cancer cell lines. Cancer Res 1997;57:3537-3547.
112. Pikula S, Hayden JB, Awasthi S, Awasthi YC, Zimniak P. the organic anion transporting ATP-ase of rat liver. J Biol Chem 1994;269:27566-27573.
113. Pikula S, Hayden JB, Awasthi S, Awasthi YC, Zimniak P. Organic anion transporting ATP-ase of rat liver. J Biol Chem 1994;269:27574-27579.
114. Fernandez-Checa JCH, Takikawa H, Hori T, Ookthens M, Kaplowitz N. Canalicular transport of reduced glutathione in normal and mutant Eisai hyperbilirubinemic rats. J Biol Chem 1992;267:1667-1673.
115. Taniguchi K, Wada M, Kohno K, et al. A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation. Cancer Res 1996;56:4124-4129.
116. Paulusma C, Oude Elferink RPJ. The canalicular multispecific organic anion transporter and conjugated hyperbilirubinemia in rat and man. J Mol Med 1997;75:420-428.
117. Jansen PLM. Molecular biology of bilirubin metabolism. Prog Liv Dis 1995;13:125-150.
118. Berg CL. The physiology of jaundice: molecular and functional characterization of the Crigler-Najjar syndromes. Hepatology 1995;22:1338-1340.
119. Erps LT, Ritter JK, Hersh JH, Blossom D, Martin NC, Owens IS. Identification of two single base substitutions in the ugt1 gene locus which abolish bilirubin uridine diphosphate glucuronosyltransferase activity in vitro. J Clin Invest 1993;564-570.
120. Bosma PJ, Goldhoorn BG, Bakker CTM, et al. Pressence of an additional TA in the TATAA box of the B-UGT1 correlates with Gilbert syndrome. Hepatology 1994;20:266A.
121. Sato HS, Aono S, Kashiwamata S, Koiwai O. Genetic defect of bilirubin UDP-glucuronosyl transferase in the hyperbilirubineamic rat (Gunn rat). J Biol Chem 1991;266:24048-24052.
122. Iyanagi T. Molecular basis of multiple UDP-glucuronosyl transferase isoenzyme deficiencys in the hyperbilirubenemic rat (Gunn rat). J Biol Chem 1991;266:24048-24052.
123. Marin JJ, Bravo P, El-mir MP, Serrano MA. ATP-dependent bile acid transport across microvillous membrane of human term trophoblast. Am J Physiol 1995;286:G685-G694.
124. Back P, Walter K. Developmental pattern of bile acid metabolism as revealed by bile acid analysis of meconium. Gastroenterology 1980;78:671-676.
125. Kimumura A, Yamakawa R, Ushijima K, et al. Fetal bile acid metabolism during infancy: analysis of 1b-hydroxylated bile acids in urine, meconium and feces. Hepatology 1994;20:819-824.
126. Watkins JB. Neonatal cholestasis: Developmental aspects and current concepts. Sem Liv Dis 1993;13:276-288.
127. Shaffer EA, Zahavi I, Gall GD. Portnatal development of hepatic bile formation in the rabbit. Dig Dis Sci 1985;30:558-563.
128. Ballatori N, Clarkson TW. Developmental changes in the biliary excretion of methylmercury and glutathione. Science 1982;216:61-63.
129. Dubuisson C, Cresteil D, Desrochers M, Decimo D, Hadchouel M, Jaquemin E. Ontogenic expression of the Na^+-independent organic anion transporting polypeptide (oatp) in rat liver and kidney. J Hepatology 1996;25:932-940.
130. Boyer JL, Hagenbuch B, Ananthanarayanan M, Suchy FJ, Stieger B, Meier PJ. Phylogenic and ontogenic expression of hepatocellular bile acid transport. Proc Natl Acad Sci 1993;90:435-438.
131. Weymann A, Ananthanarayanan M, Arrese M, et al. Differential developmental expression of hepatic canalicular ABC transporters Mrp2 and spgp. Hepatology 1997;26:291A(abst)
132. Quigly EMM, Marsh MN, Shaffer JL, Markin RS. Hepatobiliary complications of total parenteral nutrition. Gastroenterology 1993;104:286-301.

133. Hofmann AF. Defective biliary secretion during total parenteral nutrition: probable mechanisms and possible solutions. J pediatr Gastroenterol Nutr 1994;20:367-390.
134. Tuchweber B, Perea A, Lee D, Yousef IM. Lithocholic acid-induced cholestasis in new born rats. Toxicol Lett 1983;19:107-112.
135. Yousef IM, Tuchweber B. Bile acid composition in neonatal life in rats. Biol Neonate 1982;42:105-112.
136. Lewittes M, Tuchweber B, Weber AM, Roy CC, Yousef IM. resistance of the suckling guinea pig to lithocholic acid induced cholestasis. Hepatology 1984;4(3):486-491.
137. Tuchweber B, Ducruet N, Perea A, Yousef IM, Weber AM. Development of bile secretory function in the neonatal guinea pig. Biol Neonate 1990;58:279-290.
138. Teitelbaum DH. Parenteral nutrition -associated cholestasis. Curr Opin Pediatr 1997;9:270-275.
139. Das JB, Poulos ND, Ansari G. Biliary lipid composition and bile acid profiles during and after enteral fast of total parenteral nutrition in the rabbit. J pediatr Gastroenterol Nutr 1996;22:85-91.
140. Duerksen DR, van Aerde JE, Chan G, Thomson ABR, Jewell LJ, Clandinin MT. Total parenteral nutrition impairs bile flow and alters bile composition in newborn piglet. Dig Dis Sci 1996;9:1864-1870.
141. Graham MF, Tavill AS, Halpin TC, Louis LN. Inhibition of bile flow in the isolated perfused rat liver by a synthetic parenteral amino acid mixture: associated net amino acid fluxes. Hepatology 1984;4:69-73.
142. Marin JJG, Bravo P, Perez-Barriocanal F, El-mir M, Villanueva GR. Hyperglycemia-induced cholestasis in the isolated perfused rat liver. Hepatology 1991;14:184-191.
143. Shattuck KE, Grinnel CD, Rassin DK. Amino acid infusions induce reversible, dose related decreases in bile flow in the isolated perfused rat liver. JPEN 1993;17:171-176.
144. Moss RL, Das JB, Ansari G, Raffensperger JG. Hepatobiliary dysfunctioning during total parenteral nutrition is caused by infusate, not the route of administration. J Pediatr surg 1993;28:391-396.
145. Vileisis RA, Inwood RJ, Hunt CE. Prospective controlled study of parenteral nutrition-associated cholestatic jaundice: effect of protein intake. J pediatr 1980;96:893-897.
146. Lopez del Pino VH, LaRusso F. Dissociation of bile flow and biliary lipid secretion from biliary lysosomal enzyme output in experimental cholestasis. J Lipid Res 1981;22:229-235.
147. Van der Meer R, Vonk RJ, Kuipers F. Cholestasis and the interactions of sulfated glyco- and taurolthocholate with calcium. Am J Physiol 1988;254:G644-G649.
148. Hata S, Nezo R, Kubota A, Kamata S, Takagi Y, Okada A. Effect of aminoacids in total parenteral nutrition on cholestasis in newborn rabbits. J Pediatr surg 1994;29:892-895.
149. Bruck R, Haddad P, Graf J, Boyer JL. Regulatory volume decrease stimulates bile flow, bile acid excretion, and exocytosis in isolated perfused rat liver. Am J Physiol 1992;262:G806-G812.
150. Häusinger D, Lang F, Gerok W. Regulation of cell function by the cellular hydration state. Am J Physiol 1994;267:E343-E355.
151. Häusinger D. The role of cellular hydration in the regulation of cell function. Biochem J 1996;313:697-710.
152. Kakis G, Yousef IM. Pathogenesis of lithocholate- and taurolithocholate induced intrahepatic cholestasis in rats. Gastroenterology 1978;75:595-607.
153. Vonk RJ, Tuchweber B, Massé D, et al. Intrahepatic cholestasis induced by allo monohydroxy bile acids in rats. Gastroenterology 1981;80:242-249.
154. Fouin-Fortunet H, Le Quernec L, Erlinger S. Hepatic alterations during total parenteral nutrition in patients with inflamatory bowel disease: a possible consequence of lithocholate toxicity.
. Gastroenterology 1982;82:932-937.
155. Das JB, Uzoaru IL, Ghaus Ansari G. Biliary lithocholate and cholestasis during and after Total Parenteral Nutrition: an experimental study. Proc Soc Exp Biol Med 1995;210:253-259.
156. Capron JP, Gineston JL, Herve ME, Braillon A. Metronidazole in prevention of cholestasis associated with total parenteral nutrition. Lancet 1983;446-447.
157. Kubota A, Okada A, Imura K, et al. The effect of metronidazole on TPN associated liver disfunction in neonates. J Pediatr surg 1990;25:618-621.
158. Spurr SG, Grylack LJ, Mehta NR. Hyperalimentation-associated neonatal cholestasis: effect of oral gentamicin. JPEN 1989;13:633-636.
159. Freund HR, Muggia-Sullam M, LaFrance R, Enrione EB, Popp MB, Bjornson HS. A possible benificial effect of metronidazole in reducing TPN-associated liverfunction derangements. J Surg Res 1985;38:356-363.
160. Neale G, Caughey DE, Mollin DL, Booth CC. Effects of intrahepatic and extrahepatic infection on liver function. Brit Med J 1966;1:382-387.
161. Vermillion SE, Gregg JA, Baggenstoss AH, Bartholomew LG. Jaundice associated with bacteremia. Arch Intern med 1969;124:611-618.

162. Nunes GF, Blaisdell FW, Margaretten W. Mechanism of hepatic dysfunction followin shock and trauma. Arch Surg 1970;100:546-556.
163. Franson TR, Hierholtzer WJ, LaBreque DR. Frequency and characteristics of hyperbilirubinemia associated with bacteremia. Rev Infect Dis 1985;7:1-9.
164. te Broekhorst T, Urlus M, Doesburg W, Yap SH, Goris RJA. Etiologic factors of jaundice in severely ill patients. J Hepatology 1988;7:111-117.
165. Quale JM, Mandel LJ, Bergasa NV, Straus EW. Clinical significance and pathogenesis of hyperbilirubinemia associated with Staphylococcus Aureus septicemia. Am J Med 1988;85:615-618.
166. Pirovino M, Meister F, Rubli E, Karlaganis G. Preserved cytosolic and synthetic liver function in jaundice of severe extrahepatic infection. Gastroenterology 1996;96:1589-1595.
167. Blaschke TF, Elin RJ, Berk PD, Song CS, Wolff SM. Effect of induced fever on sulfobromphtalein kinetics in man. Ann Intern Med 1978;221-226.
168. Wang P, Zheng F, Chaudry H. Hepatic extraction of indocynanine green is depressed early in sepsis despite increased hepatic blood flow and cardiac output. Arch Surg 1991;126:219-223.
169. Chaudry H, Schleck S, Clemens MG, Kupper TE, Baue AE. Altered hepatocellular active transport. Arch Surg 1982;117:151-157.
170. Utili R, Abernathy CO, Aron SA, Zimmerman HJ. In vivo effects of Escherichia coli endotoxin on sulfobromophtalein clearance in the guinea-pig. Experentia 1977;33:1631-1632.
171. Utili R, Abernathy CO, Zimmerman HJ. cholestatic effects of Escherichia coli endotoxin on the isolated perfused rat liver. Gastroenterology 1976;70:248-253.
172. Trauner M, Nathanson MH, Rydberg SA, et al. Endotoxin impairs biliary glutathione and HCO_3^- excretion and blocks the choleretic effict of nitric oxide in rat liver. Hepatology 1997;25:1184-1191.
173. Whiting JF, Green RM, Rosenbluth AB, Gollan JL. Tumor necrosis factor-alfa decreases hepatocyte bile salt uptake and mediates endotoxin-induced cholestasis. Hepatology 1995;22:1273-1278.
174. Green RM, Whiting JF, Rosenbluth AB, Beier DR, Gollan JL. Interleukin-6 inhibits hepatocyte taurocholate uptake and sodium-potassium-adenosinetriphosphatase activity. Am J Physiol 1994;267:G1094-G1100.
175. Utili R, Abernathy CO, Zimmerman HJ. Inhibition of Na^+, K^+-Adenosinetriphosphatase by endotoxin: A possible mechanism for endotoxin-induced cholestasis. J Infect Dis 1977;136:583-587.
176. Hagmann WC, Hierholtzer WJ, Keppler D. Role of peptide leukotrienes and their hepatobiliary elimination in endotoxin action. Circ Shock 1984;14:223-235.
177. Roelofsen H, van der Veere CN, Ottenhoff R, Schoenmaker B, Jansen PLM, Oude Elferink RPJ. Decreased bilirubin transport in the perfused liver of endotoxemic rats. Gastroenterology 1994;107:1075-1084.
178. Roelofsen H, Schoenmaker B, Bakker CTM, Ottenhoff R, Jansen PLM, Oude Elferink RPJ. Impaired organic anion transport in endotoxemic rats. Am J Physiol 1995;269:G427-G434.
179. Trauner M, Arrese M, Soroka CL, et al. The rat canalicular conjugate export pump (mrp2) is down regulated in intrahepatic and obstructive cholestasis. Gastroenterology 1997;113:255-264.
180. Johnson WG, Basket TF. Obstetric cholestasis: a 14 year review. Am J Obstet Gyneacol 1979;133:299-303.
181. Reyes H. The enigma of cholestasis of pregnancy: lessons from Chile. Hepatology 1982;2:87-95.
182. Holzbach TR, Sivak DA, Braun WE. Familial recurrent intrahepatic cholestasis of pregnancy: A genetic stucy providing evidence for transmission of a sex-limited dominant trait. Gastroenterology 1983;85:175-179.
183. Berg B, Helm G, Petersohn L, Tryding N. Cholestasis of pregnancy. Clinical and laboratory studies. Acta Obstet Scand 1986;65:107-113.
184. Rioseco AJ, Ivankovic MB, Manzur A, et al. Intrahepatic cholestasis of pregnancy: a retrospective case-control study of perinatal outcome. Am J Obstet Gyneacol 1994;3:890-895.
185. Reyes H, Ribalta J, Gonzalez C, Segovia N, Oberhauser E. Sulfobromophthalein clearance tests before and after ethinyl estradiol administration, in women and men with familial history of intrahepatic cholestasis of pregnancy. Gastroenterology 1981;81:226-231.
186. de Pagter AGF, van Berge Henegouwen GP, ten Bokkel Huinink JA, Brandt KH. Familial benign recurrent intrahepatic cholestasis. Interrelation with intrahepatic cholestasis of pregnancy and from oral contraceptives? Gastroenterology 1976;71:202-207.
187. Davies MH, Ngong JM, Yucesoy M, et al. The adverse influence of pregnance upon sulphation: aclue to the patogenesis of intrahepatic cholestasis of pregnancy? J Hepatology 1994;21:1127-1134.
188. Rolfes DB, Ishak KG. Liver disease in pregnancy. Histopathol 1986;10:555-570.
189. Bouchard G, Yousef IM, Tuchweber B. Decreased biliary glutathione content is responsible for the decline in bile salt-independent flow induced by ethinyl estradiol in rats. Toxicol Lett 1994;74:221-233.

190. Accatino L, Figueroa C, Pizarro M, Solis N. Enhanced billiary excretion of canalicular membrane enzymes in estrogen induced and obstructed cholestasis, and effects of different bile acids in the isolated perfused rat liver. J Hepatology 1995;22:658-670.
191. Kreek MJ, Peterson RE, Sluisenger MH, Jeffries GH. Effects of ethinylestradiol induced cholestasis on bile flow and biliary excretion of estradiol and estradiol glucuronide by the rat. Proc Soc Exp Biol Med 1996;131:646-650.
192. Iqbal S, Mills CO, Elias E. Biliary permeability during ethinyl estradiol-induced cholestasis studied by segmented retrograde intrabiliary injections in rats. J Hepatology 1985;1:211-219.
193. Davis RA, Kern F, Showalter R, Sutherland E, Sinenski M, Simon FR. Alterations of hepatic Na^+/ K^+ATPase and bile flow by estrogen: effects on liver surface membrane lipid structure and function. Proc Natl Acad Sci 1978;75:4130-4134.
194. Berr F, Simon FR, Reichen J. Ethinylestradiol impairs bile salt uptake and Na^+-K^+ pump function of rat hepatocytes. Am J Physiol 1984;274:G437-G443.
195. Rosario J, Sutherland E, Zaccaro L, Simon FR. Ethinylestradiol administration selectively alters sinusoidal membrane lipid fluidity and protein composition. Biochemistry 1988;27:3939-3946.
196. Bossard R, Stieger B, O'Neil B, Fricker G, Meier PJ. Ethinylestradiol treatment induces multiple canalicular membrane transport alterations in rat liver. J Clin Invest 1993;91:2714-2720.
197. Koopen NR, Wolters H, Havinga R, et al. Impaired activity of the bile canalicular organic anion transporter (mrp2/cmoat) is not the main cause of ethinylestradiol-induced cholestasis in the rat. Hepatology 1998;27:537-545.
198. Monte MJ, Morales AI, Arevalo M, Alvaro I, Macias RIR, Marin JJG. Reversible impairment of neonatal hepatobiliary function by maternal cholestasis. Hepatology 1996;23:1208-1217.
199. Slott PA, Liu MA, Tavoloni N. Origin,patern and mechanism of bile duct proliferation following biliary obstruction in the rat. Gastroenterology 1990;99:466-467.
200. Chen J, Murray M, Liddle C, Jiang XM, Farrell GC. Down regulation of male-specific cytochrome P450s 2C11 and 3A2 in bile duct ligated male rats: importance to reduced hepatic content of cytochrome P450 in cholestasis. Hepatology 1995;22:580-587.
201. Krähenbühl S, Talos C, Lauterburg BH, Reichen J. Reduced antioxidative capacity in liver mitochondria from bile duct ligated rats. Hepatology 1995;22:607-612.
202. Rahner C, Stieger B, Landmann L. Structure-function correlation of tight junctional impairment after intrahepatic and extrahepatic cholestasis in rat liver. Gastroenterology 1996;110:1564-1578.
203. Stieger B, Meier PJ, Landmann L. Effect of obstructive cholestasis on membrane traffic and domain-specific expression of plasma membrane proteins in rat liver parenchymal cells. Hepatology 1993;20,No. 1.201-212.
204. Fricker G, Landmann L, Meier PJ. Extrahepatic obstructive cholestasis reverses the bile salt secretory polarity of rat hepatocytes. J Clin Invest 1989;84:876-885.
205. Zimmerman HJ, Blaser H, Zimmermann A, Reichen J. Effect of development on the functional and histological changes induced by bile-duct ligation in the rat. J Hepatology 1994;20:231-239.
206. Barr VA, Hubbard AL. Newly synthesized hepatocyt plasma membrane proteins are transported in transcytotic vesicles in the bile duct ligated rat. Gastroenterology 1993;105:554-571.
207. Tutois S, Montagutelli X, Da Silva V, et al. Erythropoietic protoporpheria in the house mouse. J Clin Invest 1991;88:1730-1736.
208. Boulechfar S, Lamoril J, Montagutelli X, et al. Ferrochelatase structural mutant (*Fech^{m1pas}*) in the house mouse. Genomics 1993;16:645-648.

Chapter 9

General discussion

GENERAL DISCUSSION.

In patients it is often very difficult to define the initiating event in the onset of cholestasis because complex interactions can take place in the liver as a consequence of sometimes relatively small defects. A viscious cycle may develop, in which accumulation of substrates or metabolites causes damage to hepatocytes, which gives rise to inflammation and effects on transport proteins involved in bile formation. In order to develop optimal therapy, it is important to make a distinction between the primary event and the secondary consequences. For some cases of cholestatic liver disease mutational analysis is or will shortly become available for diagnosis. In other cases one has to rely on liver function tests and serology. Differential diagnosis remains cumbersome and, as a consequence, treatment can only be palliative.

In an experimental setting, this issue is more easily addressed and cholestasis can be quantified on the basis of direct measurement of bile flow. With the molecular tools now available, changes in bile production can be related to changes in transporter activity, protein levels and gene transcription. However, even in this situation it may be difficult to separate primary events from secondary consequences. The relation between ntcp expression and activity, serum bile salt levels and actual bile formation in different experimental models is illustrated by evaluation of published data and the work described in this thesis. It has become clear that ntcp is down-regulated in a number of cholestatic models mainly at transcriptional level. The most important question in this regard concerns the reason for this down-regulation. High intracellular bile salt concentrations will damage the cell and down-regulation of bile salt uptake systems may protect the cell from this toxicity. The presence of a bile salt-responsive element in the promotor region of ntcp supports the view that the hepatocyte anticipates to high intracellular bile salt concentrations by down regulation of ntcp. We showed in our studies that this regulation does not take place during physiological fluctuations in plasma bile salts and hepatic bile salt flux under non-cholestatic conditions (Chapter 4), indicating that relatively high bile salt concentrations are needed for ntcp down-regulation.
It has been speculated that down-regulation of ntcp is an primary event in cholestasis by causing diminished bile salt uptake leading to increased bile salts in plasma and reduced bile salt secretion into bile. Simon and co-workers have suggested that the primary cause for EE cholestasis is located at the basolateral membrane and is the reason for elevated serum bile salt levels in plasma and reduced bile formation in this model. Yet, in our studies, using a lower dose of the estrogen, we did not find an increase in plasma bile salt levels, but we did observe a down-regulation of ntcp under the influence of EE (Chapter 2). Conversely, mdr2-knockout mice do not show cholestasis on bile flow, but do have elevated bile salt levels in plasma. We showed that post-transcriptional down-regulation of ntcp in mdr2 (-/-) mice possibly contributes to this phenomenon (Chapter 5). We therefore conclude that there are different levels of regulation for ntcp involving both transcriptional and post-transcriptional events. When the system in the mdr2-knockout mice is stressed by feeding cholesterol, bile salt

synthesis is increased and the mice develop a marked hyperbilirubinemia and hypercholenemia but bile flow remains unchanged (Chapter 6.). Bilirubin under there conditions is present in blood largely in unconjugated form, which may be a consequence of toxic effects of high bile salt concentrations on erythrocytes, leading to increased hemoglobin degradation. These findings underline that in situations with highly increased bile salt and bilirubin levels in plasma and down-regulation of ntcp, bile formation is not necessarily affected.

The finding that ntcp is down-regulated in EE-treated rats makes the story even more complicated. With our treatment schedule (5mg/kg, 3days), we did not find a "cholestatic" effect of the estrogen on plasma markers. Bile flow, however, was severely decreased mainly due to a decrease of the bile salt independent fraction. We tested the involvement of the plasma bile salts in down-regulation of ntcp by administration of EE in a model in which the enterohepatic circulation of bile salts is interrupted, i.e., in bile diverted rats. Ntcp was not affected by bile diversion *per se*, but was down-regulated even more strongly than in control rats when EE was administered (chapter 8). These results show that, in this particular case, probably not the bile salt concentration, either in plasma or intracellulary, is involved but that EE itself and/ or the reduction in bile flow affects ntcp expression. Down-regulation of ntcp is clearly not the most important event in this cholestatic model, because it does not affect bile salt secretion. Another example of ntcp down-regulation in a non-cholestatic model is provided by ferrochelatase-deficient mice (Chapter7). These mice show an increased bile production and biliary bile salt secretion while, at the same time, bile salts in serum are strongly increased. In these mice we found a very pronounced down-regulation of ntcp. The increased BS load into the intestine did not result in increased BS loss via feaces. As the intestinal bile salt transporter (ibst) is upregulated with increasing bile salt-loads there may be an accelerated enterohepatic circulation in these animals. The presence of this intestinal transporter in the bile duct epithelial cells probably contribute to the high serum bile salts in these mice, via an intrahepatic circulation of bile salts. These examples show that the relation between cholestatic serum markers, carrier expression, in this case of ntcp, and bile flow is not always clear-cut and different regulatory processes take place. Cause and consequence cannot be discerned directly with the current state of knowledge.

Another transport protein potentially involved in cholestasis is the canalicular organic anion transporter mrp2. This transporter is down-regulated in cholestatic models like bile duct ligation and cytokine administration and also in cell culture . In a extrahepatic cholestatic model, as is bile duct ligation, down-regulation of hepatic transporters is a consequence of the cholestasis by definition. Secretion of glutathione in bile is an important factor in the formation of the bile salt independent fraction of bile flow. In EE cholestasis the secretion of organic anions and glutathione is impaired as well as the bile salt independent fraction of bile flow. Because mrp2 is important for both the secretion of organic anions and glutathione, and mrp2 was found to be decreased in canalicular membranes isolated from EE treated rats, we questioned if mrp2 down-regulation could also be a primary event in EE-induced bile flow reduction (Chapter 2). It was found that EE treatment indeed reduced mrp2

levels in canalicular membranes in control rats. When EE was administered to mrp2-deficient rats, however a very similar reduction in bile flow was found. These experiments therefore exclude the possibility that mrp2 down-regulation is a major event in EE-induced bile flow reduction. In addition, it was found that in this model, in contrast to sepsis and bile duct ligation, mrp2 down-regulation only takes place posttranscriptionally.

Although the model of EE-cholestasis has been advocated as a clean model for studying the consecutive events in intrahepatic cholestasis caused by reduction of BSIF, the results described in this thesis show that the bile salt secretion is also affected (Chapter 3). The bile salt pool was decreased and the composition of the pool was changed when rats were treated with EE. It was shown that the neutral pathway of bile salt synthesis is inhibited and that the acidic pathway takes over, resulting in a changed bile salt composition of bile. The same experiments in long term bile diverted rats revealed that also inhibition of enzymes involved in bile formation was found, but that this was not reflected in the biliary bile salt composition. These results underline the importance of combining data on gene expression enzyme activities and actual metabolic fluxes.

The work described in this theses emphasizes that cholestasis is often multifactorial in nature and, important for clinical practice, that elevated plasma concentrations of bile salt and bilirubin do not always are indicative for impaired bile formation. It was shown that both unaffected plasma markers and decreased bile flow and increased plasma markers and unaffected bile flow can occur simultaneously. In the past few years the cloning of a number of proteins involved in hepatobiliary transport has greatly increased our knowledge of mechanisms of bile formation and disturbances herein. This knowledge has also provided the possibility to identify mutations responsible for some inherited forms of cholestasis and treatment of these patients can therefore be more directed to the primary event in the near future. For a better understanding of the cholestatic syndromes that cannot be diagnosed directly, knowledge concerning hepatic transporters involved in bile formation and their regulation under cholestatic conditions should be combined with actual effects on bile formation in order to complete the picture. Both the effects of potential inducers of cholestasis as well as treatment strategies should be evaluated in this way. The use of knock out animal models will contribute, but compensatory reactions and side effects of a missing gene may also complicate the story, as has become clear during the extensive study in the GY/TR⁻ rat in the past ten years. A more directed approach will be the use of conditional knockouts or an antisense approach in which the short term effects of a defective gene can be analyzed. Concomitantly with the increasing use and development of molecular biological techniques, the actual physiological effects should be carefully monitored.

SAMENVATTING

Cholestase wordt gedefinieerd als een verstoring in de vorming of afvoer van gal door de lever. Dit leidt tot biochemische en morfologische veranderingen in dit orgaan, waardoor galcomponenten zich zullen ophopen in het bloed. Dit laatste wordt in de kliniek gebruikt om verstoring van de galvorming te diagnostiseren. Door de ophoping van geconjugeerd bilirubine in het bloed vertonen cholestatische patienten vaak geelzucht.

De oorzaken die ten grondslag liggen aan de ontwikkeling van cholestase in patienten zijn zeer verschillend. Obstructie van de galafvoer kan worden veroorzaakt door afsluiting van de galwegen door een galsteen of een tumor. Er zijn een groot aantal geneesmiddelen die de laatste jaren zijn geïdentificeerd als potentiële veroorzakers van geelzucht. Een aantal leverziekten gaat vaak samen met cholestase en bij ernstige sepsis kan ook cholestase en geelzucht ontstaan. Verder zijn er een aantal aangeboren afwijkingen in galvorming bekend, die variëren van defecten in de synthese van galzouten uit cholesterol, tot afwezig zijn of slecht functioneren van belangrijke eiwitten die een rol spelen in de galvorming. Ondanks toegenomen kennis is het in een klinische situatie vaak erg moeilijk om de primaire oorzaken in de ontwikkeling van het cholestatische proces aan te wijzen. Er kan een vicieuze cirkel ontstaan, waarin de accumulatie van substraten en toxische metabolieten in de lever schade veroorzaakt aan levercellen, wat aanleiding geeft tot ontstekingsreacties en effecten op transporteiwitten die betrokken zijn bij de galvorming. Voor sommige aangeboren afwijkingen zal binnenkort mutatie-analyse ter beschikking komen. In alle andere gevallen moet diagnose gebaseerd worden op lever-histologische veranderingen en analyse van de bloedsamenstelling. Differentiële diagnose blijft daarom erg moeizaam en behandeling kan alleen worden gericht op symptoombestrijding zolang primaire oorzaken niet te achterhalen zijn.

In een dier-experimentele situatie wordt cholestase altijd gedefinieerd op basis van verlaging van de galproductie, een parameter die in patiënten meestal niet kan worden bepaald. In dit proefschrift wordt de relatie gelegd tussen galvorming, zoals deze gemeten wordt in verschillende pathologische proefdier-modellen, en cholestatische parameters, die in het bloed worden bepaald. De toegenomen kennis ten aanzien van membraan-gebonden transporteiwitten die in de lever een rol spelen in het proces van galvorming, verschaft de mogelijkheid om ook de regulatie van deze eiwitten onder cholestatische condities te integreren in deze studies.

Een bekende stof die cholestase kan veroorzaken in de mens en proefdiermodellen is het vrouwelijk hormoon oestrogeen. Er is een groep vrouwen die cholestase ontwikkelen tijdens de zwangerschap of tijdens het gebruik van oestrogeen bevattende anticonceptie-middelen. Over het mechanisme van dit type cholestase is nog weinig bekend. Wanneer het synthetisch oestrogeen ethinyl-estradiol (EE) aan ratten wordt toegediend (5mg/kg, 3 dagen) neemt de galproductie zeer snel af, terwijl op dat moment

nog geen veranderingen in het bloed kunnen worden aangetoond. Hoofdstuk 2 behandelt de betrokkenheid van het membraangebonden transporteiwit mrp2, dat een rol speelt in de gal-uitscheiding van organische anionen zoals geconjugeerd bilirubine, in de ontwikkeling van EE-geassocieerde cholestase. Als model hiervoor wordt de mutante GY/TR$^-$ rat gebruikt. Door een mutatie bezit deze rat geen mrp2 transporteiwit in de lever. Toediening van ethinyl-estradiol geeft eenzelfde verlaging in de galproductie in deze mutante, mrp2 deficiënte ratten, als ook bereikt wordt in EE-behandelde normale ratten. Hieruit kan geconcludeerd worden dat veranderingen in mrp2 niet de belangrijkste oorzaak zijn van dit type cholestase.

Zoals de meeste vormen cholestase is ethinyl-estradiol cholestase multifactorieel. Naast de sterke verlaging van het deel van de galproductie dat niet afhankelijk is van de uitscheiding van galzouten in de gal, is ook de uitscheiding van galzouten zelf veranderd na toediening van het oestrogeen. Van de twee belangrijkste biosynthetische routes die cholesterol in de lever omzetten in galzouten, de neutrale en de "zure" of alternatieve route, wordt de z.g.n. neutrale route geremd door ethinyl-estradiol en de alternatieve route niet. Dit werd aangetoond in levers, geïsoleerd uit behandelde ratten, op zowel activiteitsniveau, als ook op niveau van het coderende materiaal, het RNA. Deze resultaten werden echter niet weerspiegeld wanneer *in vivo* de galvorming werd bestudeerd. Dit laatste wordt waarschijnlijk veroorzaakt doordat de galzoutsynthese voor een groot deel wordt overgenomen door de alternatieve route en doordat gelijktijdig ook andere enzymen verderop in de route beïnvloedt zijn door het oestrogeen zoals aangetoond in hoofdstuk 3. Deze bevindingen tonen mede het belang aan van het combineren van gegevens over genexpressie met de daadwerkelijke metabole veranderingen.

Het belang van het relateren van veranderingen in galproductie en bloedsamenstelling aan veranderingen in transporteractiviteit, hoeveelheden transporters in de levermembraan en gentranscriptie, kan worden geïllustreerd aan de hand van de regulatie van het galzouttransporterende eiwit, ntcp. In de hoofdstukken 6 tm 8 is de regulatie van ntcp bestudeerd in verschillende modellen.

Ntcp is een Na^+-afhankelijk eiwit dat in de lever betrokken is bij de opname van galzouten vanuit het bloed. Omdat galzouten in principe toxisch zijn voor cellen, is het van belang dat de concentratie van galzouten in de lever laag gehouden wordt. Een belangrijk mechanisme voor de levercel is het reduceren van de opname van galzouten, als deze niet meer voldoende kunnen worden uitgescheiden vanuit de lever naar de gal zoals het geval is tijdens cholestase. Het gen dat codeert voor ntcp bevat een galzoutresponsief element, waardoor het gen adequaat kan reageren op hoge concentraties galzouten. In alle tot nu toe bekende cholestatische modellen wordt een verlaging van de hoeveelheid ntcp in de lever gevonden, wat hoofdzakelijk wordt veroorzaakt door verlaagde transcriptie van het coderend gen. In hoofdstuk 6 wordt aangetoond dat deze regulatie niet plaats vindt tijdens

fysiologische fluctuaties in de galzoutconcentratie in het bloed en tijdens fluctuaties in de stroom van galzouten door de lever onder niet-cholestatische condities. Dit geeft aan dat relatief hoge galzoutconcentraties noodzakelijk zijn voor het naar beneden reguleren van ntcp.

Simon en medewerkers hebben gespeculeerd dat afname van ntcp-niveau's in de lever een primaire gebeurtenis is in de verhoging van galzouten in het bloedcompartiment en in de afname van de galproductie in cholestase veroorzaakt door EE. In onze studies, waarbij een lagere dosis van het oestrogeen wordt gebruikt, vinden we echter geen veranderingen in galzoutconcentraties in het bloed, maar wel een afname van ntcp-niveau's en een verlaging van de galproductie.

In muizen waarin het mdr2 gen is uitgeschakeld, waardoor deze muizen geen lipiden meer kunnen uitscheiden in de gal, is de galproductie niet verlaagd en deze muizen zijn per definitie dan ook niet cholestatisch. Echter in het bloed zijn de galzoutconcentraties sterk verhoogd. In hoofdstuk 6 wordt aangetoond dat ntcp-activiteit, bepaald in vitro, en ntcp-niveau's in de lever van deze muizen verlaagd zijn. Dit is echter niet gereguleerd op genexpressieniveau zoals in cholestatische modellen. Wanneer het systeem onder druk wordt gezet in deze mrp2 deficiënte muizen door cholesterol toe te voegen aan het voer, neemt de galzoutsynthese toe en ontwikkelen deze muizen hyperbilirubinemie en gaan ook galzoutconcentraties in het bloed zeer sterk omhooog. De galproductie wordt echter niet beïnvloed. Bilirubine is onder deze condities, in tegenstelling tot in cholestatische situaties, voornamelijk ongeconjugeerd. Dit zou veroorzaakt kunnen worden door de toxische effecten van hoge galzoutconcentraties op rode bloedcellen, leidend tot toename van de hemoglobine-afbraak. De bevindingen in dit experiment onderschrijven dat in situaties, waarin galzout en bilirubine-concentraties in het bloed zijn toegenomen en ntcp niveau's in de lever zijn afgenomen, er niet noodzakelijkerwijs sprake hoeft te zijn van een gereduceerde galproductie door de lever.

De bevinding dat ntcp is down-gereguleerd in EE-behandelde ratten maakt het verhaal nog complexer. Met ons behandelingsschema werd geen "cholestatisch" effect van het oestrogeen op de bloedsamenstelling gevonden. De galproductie was echter sterk verlaagd. De betrokkenheid van galzouten in het bloed in de EE-geïnduceerde down-regulatie van ntcp werd getest in een model waarin geen galzouten vanuit de darm terugkeren naar de lever. In deze zogenaamde gal-afgeleide rat, wordt de gal buiten het lichaam afgeleid waardoor in het poortaderbloed geen galzouten aanwezig zijn. Ntcp niveau's in de lever werden niet door het afleiden van de gal beïnvloed, maar wanneer deze ratten werden behandeld met EE werd een down-regulatie gevonden die zelfs sterker was dan in EE-behandelde controle -ratten. Deze resultaten tonen aan dat in dit geval, niet de galzoutconcentratie in het bloed, noch galzoutconcentratie in de lever van belang zijn. In tegenstelling tot andere modellen lijkt het oestrogeen zelf, of de afname in galproductie, ntcp te beïnvloeden. De down-regulatie van ntcp in dit model lijkt echter niet de belangrijkste gebeurtenis te zijn in de ontwikkeling van de cholestase, omdat de uitscheiding van galzouten in de gal nauwelijks is beïnvloed.

Een ander voorbeeld van ntcp down-regulatie in een niet-cholestatisch model wordt gevonden in ferrochelatase-deficiënte muizen (hoofdstuk 7). Deze muizen hebben zelfs een toegenomen galproductie en galzoutuitscheiding, terwijl tegelijkertijd galzouten in het bloed extreem hoog zijn. In de levers van deze muizen werd een zeer uitgesproken down-regulatie van ntcp gevonden. De toegenomen galzoutuitscheiding via de gal in de darm gaf echter geen aanleiding tot een toegenomen galzoutverlies via de faeces. Omdat de darm-galzouttransporter (ibat) omhoog gereguleerd is in de darm van deze muizen, zal er een toegenomen enterohepatische circulatie (circulatie tussen de lever en de darm) zijn van galzouten. De aanwezigheid van deze transporter in galgang-epitheelcellen draagt, via een intrahepatische circulatie van galzouten, bij aan de zeer hoge galzoutconcentraties in het bloed.

Het onderzoek beschreven in dit proefschrift benadrukt dat cholestase een proces is waarin meerdere factoren tegelijkertijd een rol kunnen spelen. Belangrijk voor de klinische praktijk is, dat verhoogde galzout- en bilirubineconcentraties in het bloed niet altijd een indicatie zijn voor een verstoorde galproductie. In dit proefschrift wordt aangetoond, dat zowel een onveranderde bloedsamenstelling en een verlaagde galstrooom, als sterk toegenomen cholestatische parameters in het bloed met een onveranderde galproductie kunnen voorkomen. In de afgelopen jaren heeft de klonering van een toegenomen aantal eiwitten, betrokken in hepatobiliair transport, de kennis van het proces van galvorming en verstoringen hierin doen toenemen. Dit heeft geleid tot de mogelijkheid mutaties te onthullen, die verantwoordelijk zijn voor een aantal aangeboren cholestatische afwijkingen. Diagnose en behandeling in deze patiënten kunnen in de nabije toekomst gerichter plaats vinden. Voor een beter begrip ten aanzien van cholestatische syndromen die nog niet direct gediagnostiseerd kunnen worden, moet de kennis betreffende transporteiwitten in de lever en hun regulatie onder cholestatische condities gecombineerd worden met de werkelijke effecten op galvorming. Zowel de effecten van potentiële cholestase-inducerende middelen, als mogelijke behandelingsmethodes zullen op deze wijze getest moeten worden. In een tijd waarin moleculair biologische technieken in toenemende mate worden ontwikkeld en toegepast, is het belangrijk de feitelijke effecten op de fysiologie niet uit het oog te verliezen.

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Aynke